

The role of endothelial function and oxidant stress in a model of insulin resistance

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*In memory
of
Mum & Dad*

Abstract

Type 2 diabetes mellitus affects over 100 million people worldwide. It is characterized by various metabolic abnormalities such as insulin resistance, aberrant insulin secretion, hyperglycaemia and a cluster of cardiovascular risk factors, including increased oxidative stress. It is associated with microvascular complications and increased potential of macrovascular disease. The aim of the studies described in this thesis was to test the hypothesis that oxidant stress contributes to an altered vascular function and impaired insulin regulation in a pre-diabetic animal model- the obese Zucker rats.

The first objective was to develop new methods to measure endothelial function in animal disease models. Firstly, without autonomic control - the *in situ* perfused hindquarters, and secondly, with autonomic control – the *in vivo* Doppler ear blood flow.

The obese Zucker rat was shown to have increased oxidative stress, as measured by plasma 8-epi-PGF_{2α}. It also had high insulin and glucose levels and impaired glucose disposal. Obese rats also had increased agonist-induced nitric oxide-dependent endothelial responses; these were further enhanced by insulin in a macrovascular preparation, but were impaired by insulin in a resistance vessel bed.

Following dietary treatment with the antioxidants, the obese plasma insulin/glucose ratio was improved. However, vitamin E blunted the enhanced endothelial-dependent vasodilator responses, and decreased plasma levels of 8-epi-PGF_{2α}. In contrast, pro-oxidant treatment with hydroquinone and buthionine-sulphoximine impaired the plasma insulin/glucose ratio, abolished endothelial hyperactivity but increased plasma 8-epi-PGF_{2α} levels. Interestingly, fructose protected against pro-oxidant-induced increases in plasma 8-epi-PGF_{2α} levels and further increases in glucose-induced plasma insulin.

In summary the redox status in obese Zucker rats was modified with antioxidant and pro-oxidant treatment. This resulted in compensatory changes in glucose disposal and endothelial function. Impaired endothelial function may initiate “damage” especially in those individuals susceptible to syndrome X, leading to insulin insensitivity and vascular dysfunction in type 2 diabetes.

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Publications

The following paper and abstracts have been published in support of this thesis

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Abbreviations

ACh	Acetylcholine
ADP	Adenosine diphosphate
AGE	Advanced glycation end products
ANS	Autonomic nervous system
ATP	Adenosine triphosphate
BH ₄	Tetrahydrobiopterin
BHT	Butylated hydroxytoluene
BPM	Beats per minute
BSA	Bovine serum albumin
BSO	L-buthionine-(S,R)-sulphoximine
Ca ²⁺	Calcium
CaCl ₂	Calcium chloride
CHCl ₃	Chloroform
cAMP	Cyclic adenine mono phosphate
cGMP	Cyclic guanine monophosphate
CNS	Central nervous system
CO ₂	Carbon dioxide
COX	Cyclooxygenase
DAG	Diacylglycerol
DNA	Deoxyribonucleic acid
EDHF	Endothelium-derived hyperpolarising factor
EDRF	Endothelium-derived relaxing factor
eNOS	Endothelial nitric oxide synthase
Fe ²⁺	Iron -reduced
Fe ³⁺	Ferric iron
FAD	Flavin adenine dinucleotide
FMN	Flavin mononucleotide
GC-MS	Gas chromatography-mass spectrometry
GFR	Glomerular filtration rate
GLUT	Glucose transporter
GSH	Reduced glutathione
GPx	Glutathione peroxidase
HbA1c	Glycosylated haemoglobin A1c
H ₂ O ₂	Hydrogen peroxide
HQ	Hydroquinone
HR	Heart rate
11β-HSD	beta-hydroxysteroid dehydrogenase
ICAM	Inflammatory cell adhesion molecule

IL	Interleukin
iNOS	Inducible nitric oxide synthase
IP ₃	Inositoltriphosphate
IRS	Insulin receptor substrate
K ⁺	Potassium
KCl	Potassium chloride
KOH	Potassium hydroxide
LDL	Low density lipoprotein
L-NAME	N ^G -nitro-L-arginine methyl ester
L-NMMA	N ^G -monomethyl-L-arginine
M ₁	Muscarinic type 1
M ₂	Muscarinic type 2
MAP	Mean arterial pressure
MDA	Malondialdehyde
MeOH	Methanol
MgCl	Magnesium chloride
MgSO ₄	Magnesium sulphate
mRNA	Messenger ribonucleic acid
NaCl	Sodium chloride
Na ⁺	Sodium
N ₂	Nitrogen
NADPH	Nicotinamide adenine dinucleotide phosphate
NADH	Nicotinamide adenine dinucleotide
NADP ⁺	Reduced nicotinamide adenine dinucleotide phosphate
NAD ⁺	Reduced nicotinamide adenine dinucleotide
NaH ₂ PO ₄	Sodium phosphate
NaHCO ₃	Sodium carbonate
NO	Nitric oxide
NO ₂	Nitrite
NO ₃	Nitrate
NOS	Nitric oxide synthase
NPY	Neuropeptide Y
NGF	Nerve growth factor
O ₂	Oxygen
O ₂ ⁻	Superoxide
OH [•]	Hydroxyl radical

ONOO ⁻	Peroxynitrite	RAGE	Receptors for AGE
PBS	Phosphate buffered saline	ROS	Reactive oxygen species
PCV	Packed cell volume	SNP	Sodium nitroprusside
<i>pD</i> ₂	Negative log ₁₀ of the concentration eliciting the half-maximal response	SOD	Cu/Zn-superoxide dismutase
PG	Prostaglandin	STZ	Streptotocin
PGI ₂	Prostacyclin	TAOS	Total antioxidant status
Phe	Phenylephrine	TBARS	Thiobarbituric acid reactive substances
PKC	Protein kinase C	TNF	Tumour necrosis factor
PLC	Phospholipase C	TXA ₂	Thromboxane A ₂
PP	Perfusion pressure	VCAM	Vascular cell adhesion molecule
PPAR	Peroxisome proliferator activated receptor	VEGF	Vascular endothelial growth factors
PSS	Physiological salt solution	VSM	Vascular smooth muscle

Units

AUC	Area under the curve	n	Nano
cm	Centimetre	pH	-log [H ⁺]
g	gram	s	Second
Hg	Mercury	s.e.mean	Standard error of mean
hr	Hour	v	Volume
i.p	Intra peritoneal	μ	Micro
i.v	Intravenous	V	Volt
k	Kilo	w	Weight
l	Litre	%	Percentage
M	Molar (moles/litre)	±	Plus or minus
m	Milli	°C	Degrees centigrade
min	Minute		
mm	Millimetre		

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Chapter 1

General Introduction

1.1 DIABETES MELLITUS

Diabetes mellitus is a metabolic disorder affecting many millions of people worldwide. It is characterised by hyperglycaemia, glycosuria, polyuria, polydipsia and ketosis. The underlying fundamental nature of the disease is a relative or absolute deficiency of insulin activity, arising from an abnormality in the islets of Langerhans.

Diabetes mellitus is classified into 2 groups. Type 1 develops in childhood it is characterised by a rapid onset of a severe syndrome. It is autoimmune in nature and occurs when β -cells are destroyed and there is severe or an absolute insulin deficiency (Castano & Eisenbarth, 1990). The causes of type 1 diabetes are complex and still not completely understood. Type 1 diabetics are thought to have an inherited, or genetic, predisposition to the disease. It is theorized that this genetic predisposition may remain dormant until it is activated by an environmental trigger such as a virus or a chemical. This starts an attack on the immune system that results in the eventual destruction of the insulin producing β -cells.

1.1.1 Type 2 diabetes

Type 2 diabetes is a condition affecting over 100 million people worldwide and accounts for 90-95 % of all diagnosed diabetic cases. This number is expected to double over the next 25 years. It develops later in life and is characterised by a gradual onset. Patients may have a long pre-diabetic phase with no glucose intolerance. This metabolic disorder is characterised by chronic hyperglycaemia and disturbances in carbohydrate, fat and protein metabolism. The disease typically exhibits defect(s) in insulin secretion and in many cases insulin resistance. Eventually the pancreatic β -cells fail and low insulin secretion results in hyperglycaemia. Hyperglycaemia can then further progress the pre-existing β -cell

dysfunction and more importantly increase the potential of macrovascular disease (Fuller *et al.*, 1983) and microvascular complications (WHO, 1999).

The genetic factors predisposing an individual to type 2 diabetes are still poorly understood. Direct evidence of genetic insulin abnormalities from single point mutations, such as Leprechaunism or Rabson-Mendenhall syndrome, are usually rare and severe. Studies in animal models have shown that the majority of defects are multiple. For example, the knockout mouse heterozygous for the insulin receptor or insulin receptor substrate-1 (IRS-1) is not insulin resistant yet mice heterozygous for both the insulin receptor and IRS-1 are insulin resistant (Consensus development conference on insulin resistance, 1998). Indirect evidence for genetic susceptibility to type 2 diabetes comes from family and ethnic assessments. Pima Indians in Arizona have the highest prevalence in world (~35 %), while Melanesians in New Guinea demonstrated low prevalence (~0%) (King & Zimmet, 1988). The strongest evidence comes from identical twin studies where there is almost complete concordance for type 2 diabetes in monozygote twins (Newman *et al.*, 1987; Medici *et al.*, 1999). The prevalence of type 2 diabetes in developing countries has given rise to the thrifty gene hypothesis (Neel, 1962). It is thought that the genetic trait which enables an individual to store and mobilise energy efficiently during times of hunting and gathering may improve survival in times of famine. However these genetic traits combined with today's sedentary lifestyles and increased dietary fat and carbohydrates, leads to obesity. And indeed as the risk factors – age, obesity, sedentary lifestyle and intra-abdominal fat increase, they correlate to the development of type 2 diabetes (Banerji *et al.*, 1995). More recent studies have suggested a thrifty phenotype hypothesis (Hales *et al.*, 1992), as poor foetal and early post-natal nutrition results in impaired long-term development of the endocrine pancreas and a greater susceptibility to type 2 diabetes. Gestational diabetes sometimes called type 4 diabetes is a condition where glucose intolerance is first recognised during pregnancy. However, in most patients it cannot be established if the

onset of diabetes occurs prior to or during pregnancy. Gestational diabetes is thought to develop in response to the production of the placental hormones progesterone (Branisteanu & Mathieu, 2003), cortisol, prolactin, human placental lactogen, and oestrogen as they have been shown, in animal models, to influence β -cell function and/or the peripheral tissue sensitivity to insulin (Kuhl, 1998). There is also an increased risk of hypertension and pre-eclampsia especially when the diabetes is poorly controlled. Recent studies have shown a correlation with the future development of type 2 diabetes (Verma *et al.*, 2002) in women who previously had gestational diabetes. Type 3 diabetes according to etiologic classification includes many specific types of diabetes, such as genetic defects of β -cell function or insulin action, diseases of the exocrine pancreas, endocrinopathies, drug or chemically-induced changes, infection-induced changes, and other genetic syndromes sometimes associated with diabetes (Report of the Expert Committee on the Diagnosis and Classification of Diabetes Mellitus, 2000).

1.1.2 Syndrome X

Syndrome X is a metabolic disorder where a number of type 2-diabetes risk factors including obesity, hypertension, glucose intolerance and hyperinsulinemia are clustered together (Reaven, 1988). It has been proposed that syndrome X may reflect a number of abnormalities or just a single abnormality. It is not known if insulin resistance is a consequence of the other disorders such as hypertension or hyperlipidaemia rather than itself a cause. Insulin resistance and /or hyperinsulinaemia have been shown to be predictors of type 2 diabetes. There is also evidence for a neurohormonal mechanism, including an endocrine role for adipocytes (Chan *et al.*, 2001). The distribution and deposition of adipose tissue is under the control of pituitary hormones such as the sex steroids, growth hormone, and the pituitary-adrenal axis. Interestingly, studies have suggested that increased prevalence of psychosocial stress factors is associated with visceral

distribution of body fat and is associated with changes in the pituitary hormone levels (Bjorntorp, 1991).

The obese Zucker has the clustered abnormalities characteristic of syndrome X and can therefore be used as a model of insulin resistance and of the pre-diabetic state (Maher *et al.*, 1995). It is also therefore a good model to study insulin resistance and the effects of oxidant stress on endothelial function. The use of diet and pharmacological interventions was studied to improve and further impair vascular function in this thesis as the decline of endothelial function may contribute to early disease and is certainly involved in frank disease complications.

1.2 INSULIN

Insulin is composed of 2 peptide chains (A-chain, 21 amino acids and B, 30 amino acids) linked by 2 disulphide bridges. Proinsulin is synthesised in the β -cells of the islets of Langerhans in the pancreas. It is then cleaved to form insulin and the inactive C-peptide. Uncleaved proinsulin, insulin and the C-peptide are packed into granules and released together into the circulation. Proinsulin has about 10% of the biological activity of insulin whereas the C-peptide is inactive. The half life of insulin in the plasma is no more than a few minutes. Most tissues have the ability to destroy insulin by enzymatic disruption. The enzyme glutathione insulin transhydrogenase catalyses the reduction and cleavage of disulphide bonds, liberating the separate A and B chains of insulin. At the same time reduced glutathione and NADPH are oxidised to glutathione and NADP.

The major stimulus for insulin synthesis and secretion is the elevation of blood glucose concentration as the islet cells are freely permeable to glucose. The action of glucose on insulin secretion is biphasic – first pre-formed insulin is rapidly secreted followed by a

slower prolonged response, which is dependent on *de novo* insulin synthesis (DeFronzo *et al.*, 1979). This acute first phase of insulin secretion is reduced or absent in diabetic patients (Brunzell *et al.*, 1976). Interestingly, studies using human β -cells *in vitro* have shown that their incubation with free fatty acids causes impairment of this first phase response (Lupi *et al.*, 2002). The release of insulin can also be stimulated by fructose. More importantly, orally ingested food is a greater stimulus for insulin secretion, due to gastrointestinal hormones release which potentiates secretion. The β -cells are innervated with noradrenergic and cholinergic nerve fibres and adrenaline and noradrenaline act on α -adrenoceptors to inhibit insulin release. It is thought that neural control is involved in the fine control of insulin release.

Insulin is a powerful pleuripotent anabolic hormone that binds a highly regulated and specific glycoprotein receptor present on the cell surface. Following binding, the receptor is autophosphorylated on multiple tyrosine residues. This causes activation of receptor kinase and tyrosine phosphorylation of a family of insulin receptor substrate (IRS) proteins. The IRS proteins then produce a series of protein-protein interactions that transmit signals from the receptor to cascade into the cell compartments. The final cellular effects being glucose uptake into all types of muscle, adipose tissue, leukocytes, fibroblasts, the lens of the eye, the aqueous humour, the pituitary gland and the liver. The glucose taken up under the influence of insulin is either utilised as fuel or converted to glycogen (Fig. 1.1). Insulin also promotes the uptake of K^+ , amino acids and stimulates protein synthesis particularly in skeletal muscle. The action of insulin on adipocytes allows the uptake of glucose which is then available for synthesis into fatty acids and glycerol-3-phosphate and thence into triglycerides. However insulin also depresses lipolysis and consequently free fatty acid release. In addition, in response to insulin, adipose tissue in turn, releases various modulators of insulin secretion and insulin action, including leptin, tumour necrosis factor- α (TNF- α), resistin, and the more recently identified adiponectin (Saltiel, 2001).

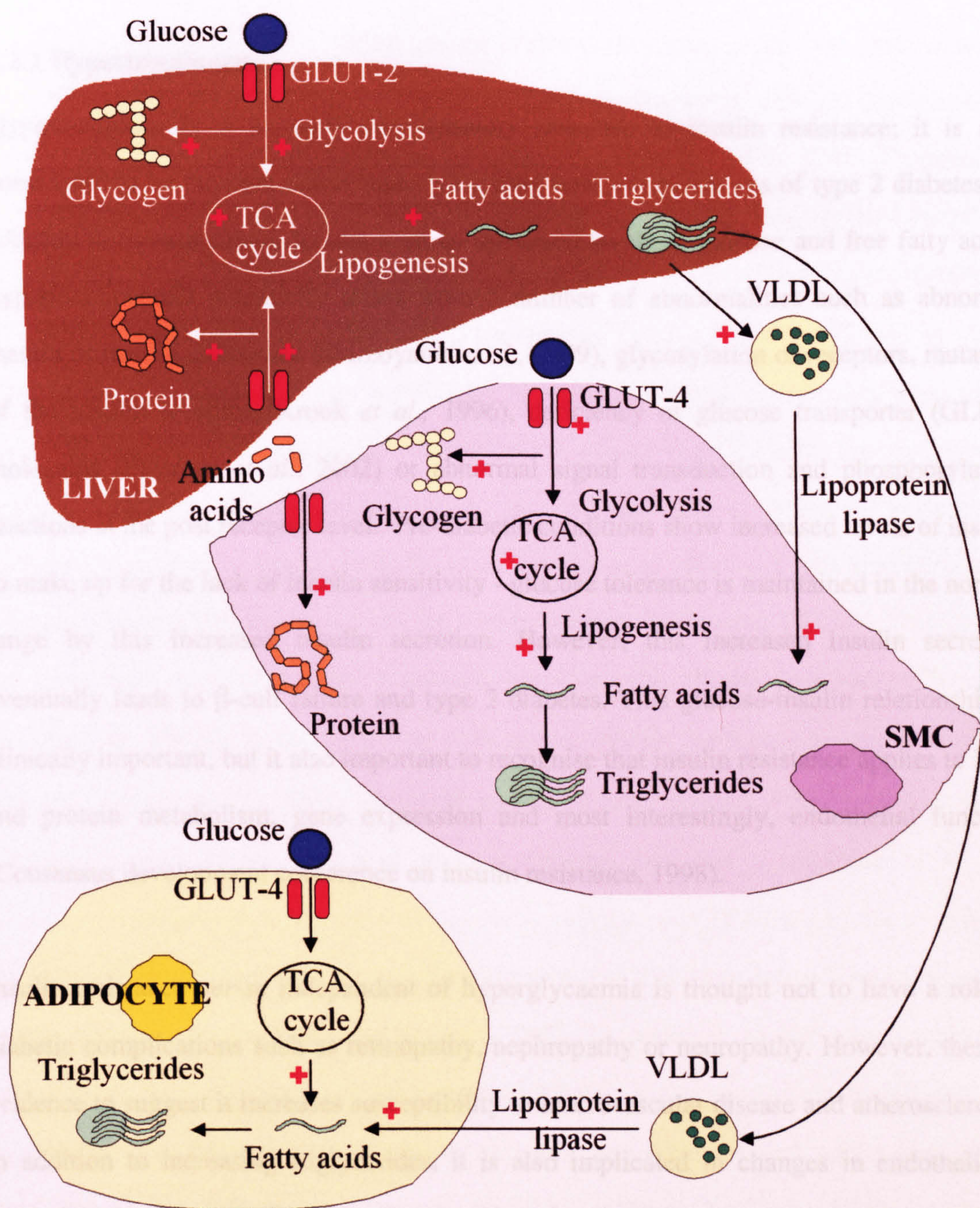


Figure 1.1 Metabolic effects of insulin in the liver, smooth muscle cells (SMCs) and adipose tissue. The illustration is adapted from [flesh and bones.com](http://fleshandbones.com) Baynes: Medical Biochemistry.

1.2.1 Hyperinsulinaemia

Hyperinsulinaemia is an early compensatory response to insulin resistance; it is also considered to be the core defect underlying long term complications of type 2 diabetes. In addition to environmental factors such as increased levels of glucose and free fatty acids, hyperinsulinaemia potentially arises from a number of abnormalities such as abnormal insulin or insulin antibodies (Shimoyama *et al.*, 1989), glycosylation of receptors, mutation of the insulin receptor (Krook *et al.*, 1996), deficiency of glucose transporter (GLUT) molecules (Petersen *et al.*, 2002) or abnormal signal transduction and phosphorylation reactions at the post receptor level. Pre-diabetic conditions show increased levels of insulin to make up for the lack of insulin sensitivity - glucose tolerance is maintained in the normal range by this increased insulin secretion. However, this increased insulin secretion eventually leads to β -cell failure and type 2 diabetes. This glucose-insulin relationship is clinically important, but it is also important to recognise that insulin resistance applies to lipid and protein metabolism, gene expression and most interestingly, endothelial function (Consensus development conference on insulin resistance, 1998).

Insulin resistance *per-se* independent of hyperglycaemia is thought not to have a role in diabetic complications such as retinopathy, nephropathy or neuropathy. However, there is evidence to suggest it increases susceptibility to macrovascular disease and atherosclerosis. In addition to increasing triglycerides, it is also implicated in changes in endothelium-dependent function, which will be discussed later in this chapter.

Studies in individuals have shown that type 2 diabetes indices can be improved with insulin sensitizer drugs such as Metformin or thiazolidinediones. Metformin enhances the action of insulin, causing suppression of hepatic glucose output and improvement in peripheral glucose utilisation. The precise mechanism of action of metformin remains unclear, but

may be by interruption of the mitochondrial oxidative processes in the liver and correction of abnormalities of intracellular Ca^{2+} metabolism in insulin-sensitive tissues (liver, skeletal muscle, and adipocytes) and cardiovascular tissue (Kirpichnikov *et al.*, 2002). Troglitazone is a thiazolidinedione, an insulin sensitizer, and enhances the insulin-stimulated peripheral glucose disposal. Troglitazone acts on the peroxisome proliferator activated receptor γ (PPAR γ), a member of the nuclear-hormone-receptor family (PPAR α , δ , and γ). After binding, the activated complex translocates to the nucleus and binds cognate promoter elements, that in turn upregulate expression of genes. How PPARs improve insulin sensitivity remains unclear but is likely to involve regulation of genes involved in the increased hydrolysis of triglycerides, decreased synthesis of fatty acids and triglycerides, as well as increased glucose uptake via the insulin sensitive transporter GLUT4. Studies into the molecular basis of insulin resistance have recently shown two loss-of function mutations of PPAR γ to be associated with severe insulin resistance in type 2 diabetes patients (Barroso *et al.*, 1999). However studies in heterozygous PPAR γ +/- mice show decreased insulin levels, enhanced glucose disposal rate and a reduction in PPAR γ receptors in peripheral and hepatic tissues. This suggests that PPARs may act to dampen the action of insulin and promote insulin resistance and that thiazolidinediones may act as partial agonists or antagonists (Olefsky, 2000). In addition to the pharmacological interventions there are many natural ligands of PPARs. Endogenous ligands include fatty, arachidonic and linoleic acids, many eicosanoids, and oxidised and native low density lipoprotein (LDL). Exogenous activators of PPAR include thiazolidinediones (increases insulin sensitivity), fibrates (lowers cholesterol) and indomethacin (anti-inflammatory). As PPAR has a large binding pocket and has many low affinity endogenous ligands, it has been proposed that PPAR may be acting as a lipid sensor.

The function of insulin receptors present on the endothelium is also unclear and they were initially thought to facilitate the transport of insulin across the vascular barrier and into the

subendothelial space (Bar *et al.*, 1978). However insulin also regulates endothelial-derived mediators involved in vasodilation, mitogenesis and, more controversially, vasoconstriction. Many studies have now demonstrated a vasodilator action by insulin (Liang *et al.*, 1982; Baron *et al.*, 1993) through the stimulated release of nitric oxide (NO) by the endothelium (Steinberg *et al.*, 1994). However insulin has also been shown to induce vasoconstriction (Edwards & Tipton, 1989), through both sympathetic mediators (Henrion & Laher, 1994), and via the release of endothelin-1 from endothelial cells (Hattori *et al.*, 1991). Insulin may also increase blood pressure by prompting salt and water retention via sympathetic nervous system.

1.3 THE ENDOTHELIUM

The vascular endothelium is an endocrine organ that maintains blood flow by regulation of vascular tone. It was once thought to be a simple barrier between blood borne substances and the underlying smooth muscle cells. However it is now known to be important in control of local homeostasis (prevention of fibrinolysis, platelet activation and inhibition, thrombogenesis) and maintenance of smooth muscle contractile phenotype. Vasoregulation by the endothelium is induced by detection of receptor ligands, such as neurotransmitters from sympathetic and non-adrenergic non-cholinergic innervation; or systemically circulating substances, such as catecholamines; or local autocooids, such as kinins. In addition, mechanical stimulation, such as shear stress evoked by blood flow, induces endothelium-dependent vasodilation. The endothelium is a facilitator of insulin transport from the circulation to vascular smooth muscle cells but more importantly mediates an insulin-induced vasodilation, a function shown to be reduced in type 2 diabetic patients (Karasu & Altan, 1993).

The endothelium mediates vasoregulation through the release of substances including prostaglandin's, endothelin's, endothelium-derived hyperpolarising factor(s) (EDHFs) (Moncada & Vane, 1979; Rubanyi *et al.*, 1985; Rubanyi & Vanhoutte, 1985) and, most importantly, NO (Furchgott & Zawadski, 1980; Palmer *et al.*, 1987). NO, prostacyclin and EDHF regulate local blood flow to regional organs and microcirculation and thereby contribute to the overall regulation of vascular tone (Moncada *et al.*, 1988). Endothelin acts to increase the overall tone of the vasculature in opposition to NO and can contribute to regulatory mechanism that can control the release of vasoactive substances including NO.

Studies of vascular dysfunction in type 2 diabetes show impaired NO (Williams *et al.*, 1996) and prostacyclin (Umeda *et al.*, 1989; Jennings, 1994) mediated vasodilation as well as increased vasoconstriction by endothelin (Laurenti *et al.*, 1997). In addition, studies in the obese Zucker rat *in vitro* have shown impaired EDHF vasorelaxation (Kaw *et al.*, 1999). The endothelium derived vasodilators NO, prostanoids and EDHF will be discussed in more detail below as they have been shown to be important mediators that are affected by diabetes.

1.3.1 Nitric Oxide (NO)

In 1980 Furchgott and Zawadski discovered that the endothelium released a factor that caused relaxation of the underlying vascular smooth muscle. It was later in 1987 this endothelium derived relaxing factor (EDRF) was identified as NO (Palmer *et al.*, 1987). NO is a potent biological modulator acting as a vasodilator, neurotransmitter and anti-microbial effector molecule depending on the cell type and situation in which it is produced. Many cell types have been shown to produce NO including macrophages, hepatocytes, epithelial cells, smooth muscle cells, cardiac myocytes, neurons and endothelial cells (Salter *et al.*, 1991).

NO is continually released from the endothelium, and contributes to a basal vascular tone (Moncada *et al.*, 1988). The shear stress on the endothelium evoked by blood flow, and receptor ligand stimulation (such as acetylcholine (ACh) or bradykinin) stimulates NO-mediated vasodilation (Fig. 1.2). Endothelial stimulation causes an influx of intracellular Ca^{2+} (see review Furchgott, 1984) that triggers the activity of a Ca^{2+} /calmodulin-dependent (Busse & Mulsh, 1990, Lopez-Jaramillo *et al.*, 1990) constitutive enzyme NO synthase (eNOS) (Palmer *et al.*, 1989 & Pollock *et al.*, 1991). NOS enzyme contains two distinct structural domains with oxygenase and reductase activity. The oxygenase contains heme, while the reductase domain binds flavin mononucleotide (FMN), flavine adenine dinucleotide (FAD), and nicotinamide adenine dinucleotide phosphate (NADPH). Linking these domains is a binding site for the cytosolic protein calmodulin. When intracellular Ca^{2+} increases, calmodulin associates with eNOS thereby facilitating electron flow between the two regions. Localisation of electron in the oxygenase domain enables the transfer of electrons to other substrates such as O_2 leading to formation of $\text{O}_2^{\cdot-}$ rather than NO (Vasquez-Vivar *et al.*, 2003). However in the presence of tetrahydrobiopterin (BH_4) a ubiquitous metabolite that serves as a NOS cofactor, the enzyme binds L-arginine cleaving the terminal guanidino nitrogen atom (Palmer *et al.*, 1988) in the presence of molecular oxygen to form NO and the co-product L-citrulline (Palmer & Moncada, 1989). NO can then diffuse rapidly over short distances into neighbouring cells to exert its effect. NO is the endogenous activator of the enzyme soluble guanylyl cyclase (Murad *et al.*, 1978), which via generation of cyclic cGMP (Ignarro *et al.*, 1987) increases extrusion of Ca^{2+} from the cytosol and decreases the proportion of myosin light chain-dependent myosin activation (Hathaway *et al.*, 1985), resulting in vasorelaxation. Additionally NO can activate K^+ channels (Bolotina *et al.*, 1994) to cause vasorelaxation through a hyperpolarising mechanism (Cowan *et al.*, 1993; Bolotina *et al.*, 1994).

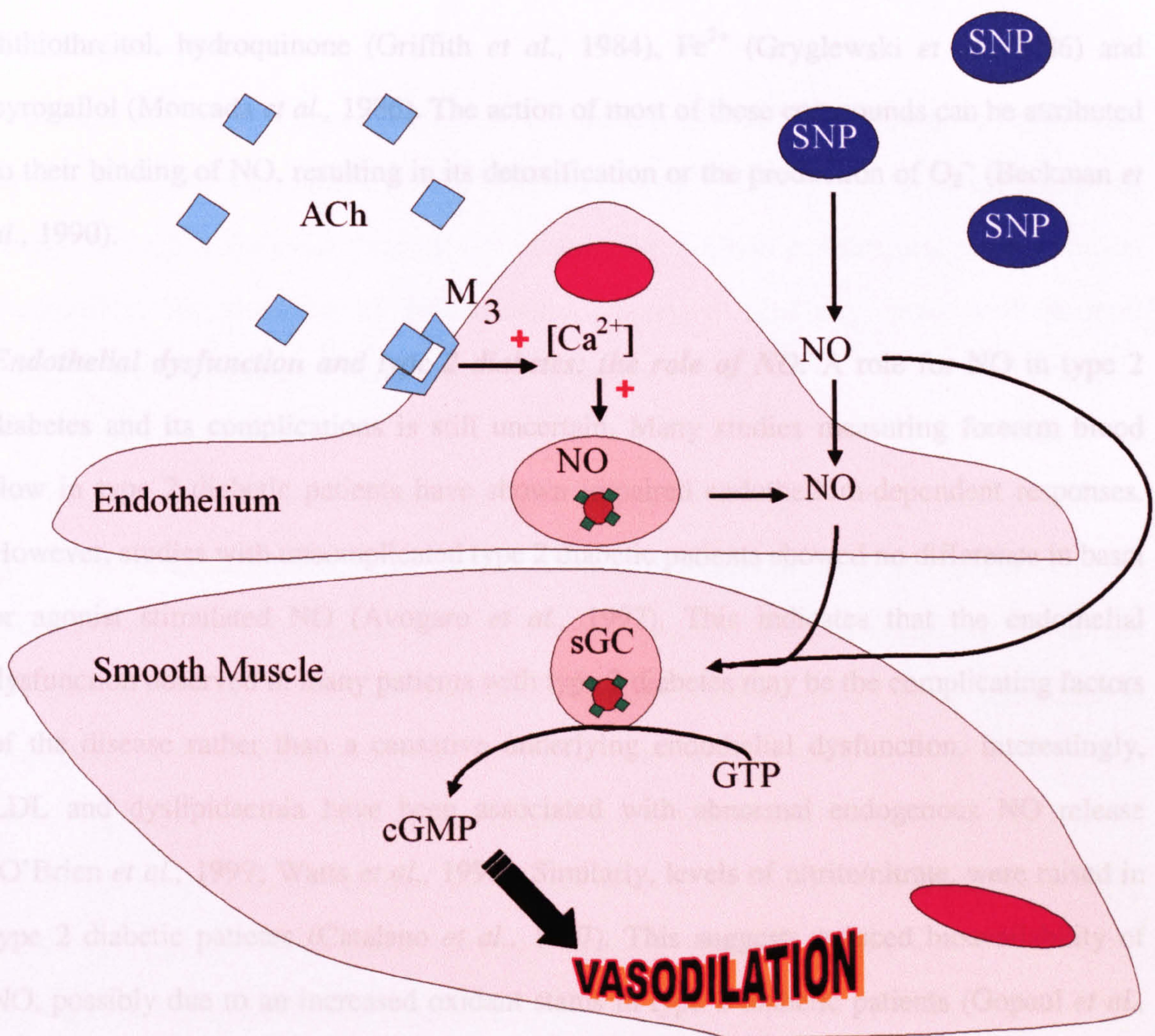


Figure 1.2 The nitric oxide pathway. Stimulation of the Muscarinic-3 (M₃) receptors with acetylcholine (ACh) leads to the increase in intracellular Ca²⁺ which in turn stimulates NOS to produce NO. Alternatively sodium nitroprusside (SNP) donates NO to directly stimulate soluble guanylyl cyclase (sGC) in the smooth muscle cells.

The half life of NO is a few seconds, before it is oxidised to the stable products nitrite (NO₂) and nitrate (NO₃), and then excreted. In plasma and water, NO is oxidised to NO₂ which is stable for hours, whereas in whole blood NO₂ is rapidly converted to NO₃. Thus, basal NO₂ levels in human blood are low (3 µM) and NO₃ levels much greater (30 µM). NO is can also be sequestered by a diverse group of compounds including haemoglobin,

dithiothreitol, hydroquinone (Griffith *et al.*, 1984), Fe^{2+} (Gryglewski *et al.*, 1986) and pyrogallol (Moncada *et al.*, 1986). The action of most of these compounds can be attributed to their binding of NO, resulting in its detoxification or the production of $\text{O}_2^{\cdot-}$ (Beckman *et al.*, 1990).

Endothelial dysfunction and type 2 diabetes: the role of NO. A role for NO in type 2 diabetes and its complications is still uncertain. Many studies measuring forearm blood flow in type 2 diabetic patients have shown impaired endothelium-dependent responses. However, studies with uncomplicated type 2 diabetic patients showed no difference in basal or agonist stimulated NO (Avogaro *et al.*, 1997). This indicates that the endothelial dysfunction observed in many patients with type 2 diabetes may be the complicating factors of the disease rather than a causative underlying endothelial dysfunction. Interestingly, LDL and dyslipidaemia have been associated with abnormal endogenous NO release (O'Brien *et al.*, 1997; Watts *et al.*, 1996). Similarly, levels of nitrite/nitrate, were raised in type 2 diabetic patients (Catalano *et al.*, 1997). This suggests reduced bioavailability of NO, possibly due to an increased oxidant status in type 2 diabetic patients (Gopaul *et al.*, 1995). In contrast, blood flow studies in animals with early diabetes show an increased formation of endothelial-derived NO (Corbett *et al.*, 1992; Tilton *et al.*, 1993). Studies in obese Zucker rats have shown enhanced endothelial-dependent relaxation in young rats (8-13 weeks) (Sexl *et al.*, 1995; Laight *et al.*, 1998; Andrews *et al.*, 2000). However, this enhancement diminishes with age (Sexl *et al.*, 1995). These studies can be explained by overcompensation of the NO pathway early in the disease process. The overcompensation then either diminishes or the production of reactive oxygen species (ROS) increases, leading to less NO synthesis or greater NO quenching. Currently there is no clear evidence of enhanced basal synthesis of endothelial-derived NO in humans (Sobrevia & Mann, 1997).

1.3.2 Prostanoids

Prostanoid is a general term used for the compounds derived from prostanoic acid. These include prostaglandins, prostacyclin and thromboxanes. This large family of mediators has diverse biological activities, ranging from regulation of blood pressure and smooth muscle contractility, to mediation of inflammatory responses. Similarly, prostanoid receptor specificity and regulatory functions are also complex.

The production of vascular prostaglandins is by *de novo* synthesis following binding of agonists such as bradykinin, histamine, angiotensin II, thrombin and even cytokines to their respective cognate receptor. Initially, arachidonic acid is removed from the membrane phospholipids by the enzyme phospholipase A₂ (Lands & Samuelsson, 1968; Vonkeman & Van Dorp, 1968). Metabolism of free arachidonic acid is then initiated through the cyclooxygenase (COX) pathway (Needleman *et al.*, 1986), which catalyses the oxygenation and peroxidation reactions of free arachidonic acid to first create PGG₂ and then PGH₂. COX has 2 catalytic components; bis-oxygenase and hydroperoxidase, which catalyse the formation of PGG₂ and PGH₂ respectively.

Endothelial dysfunction and type 2 diabetes: the role of prostanoids. The vasodilator prostacyclin (PGI₂) and the vasoconstrictor thromboxane (TxA₂) have been implicated in endothelial dysfunction associated with type 2 diabetes. PGI₂ is a potent vasodilator generated mainly by the endothelium. It acts via the inhibition of adenylyl cyclase to increase levels of cAMP. In smooth muscle this leads to phosphorylation of cAMP-dependent protein kinase, thereby inactivating the myosin light chain kinase required for contraction. The prostanoid balance is impaired in type 2 diabetes, where levels of PGI₂ are reduced (Umeda *et al.*, 1989), and TxA₂ levels are increased.

1.3.3 Endothelium derived hyperpolarising factor (EDHF)

In 1988 Taylor and Weston suggested that there was an additional factor involved in endothelial-dependent relaxation other than EDRF. This factor was termed EDHF as it caused relaxation by increasing the membrane potential of the muscle cells. Bioassay experiments suggested that EDHF acted by opening K^+ channels in vascular smooth muscle, to cause hyperpolarisation (Mombouli *et al.*, 1996). EDHF is a diffusible factor whose identity is still controversial. Indeed it may represent a family of factors including NO. Non-prostanoid products of the metabolism of arachidonic acid remain particularly appealing candidates as EDHFs because as products of phospholipase A_2 they would be expected to be released in response to elevated endothelial cell Ca^{2+} levels. Specifically, the three major candidates are the epoxyeicosatrienoic acids (cytochrome P450 metabolites of arachidonic acid), K^+ itself and H_2O_2 . Electrical coupling through myoendothelial gap junctions serves to conduct electrical changes from the endothelium to the smooth muscle and may mediate or propagate hyperpolarisation.

Vascular smooth muscle cell hyperpolarisation may be initiated by several autacoids and hormones including ACh, histamine, bradykinin, substance P, ADP, and endothelin. Interestingly, the hyperpolarisation of the smooth muscle caused by EDHF usually precedes and is more transient than the accompanying relaxation induced by other endothelium-dependent mediators. Endothelial cells themselves are also hyperpolarized by agents that cause endothelium-dependent hyperpolarisation, an event that has been attributed to the opening of Ca^{2+} -dependent K^+ channels on the endothelial cell membrane in response to the increase in cell Ca^{2+} .

Electrophysiological studies show that endothelium-dependent hyperpolarisation of vascular smooth muscle cannot be prevented by NOS and COX inhibition (Feletou & Vanhoutte, 1996; Mombouli & Vanhoutte, 1997). Studies of large arteries have shown that

membrane repolarization in the presence of NOS and COX inhibitors accounts for 20-25 % relaxation. However, studies in smaller arteries show EDHF may be responsible for up to 80 % of the total relaxation potential. In human coronary vessels and animal vessels it has been shown that the importance of EDHF increases as the arterial diameter decreases in size (Urakami-Harasawa *et al.*, 1997). This suggests that EDHF has a role in peripheral vascular resistance and local haemodynamics.

Endothelial dysfunction and type 2 diabetes: the role of EDHF. Elevated NO has been shown to induce an impairment of the EDHF pathway (Bauersachs *et al.*, 1996) through a cGMP-dependent mechanism. The observed decrease of EDHF-mediated dilation by NO would suggest that under conditions of impaired NO release there would be an enhanced formation of EDHF. Therefore, in pathophysiological states such as hypercholesterolemia, hypertension, arteriosclerosis, and diabetes, which are associated with decreased bioavailability of endothelium-derived NO, EDHF formation may be of much greater importance than under physiological conditions. Indeed, in a rabbit model of hypercholesterolemia, endothelium-dependent hyperpolarisation, mediated via opening of Ca^{2+} -activated K^+ channels, maintains endothelium-dependent dilation in response to ACh prior to development of atherosclerosis. Moreover, in the same model, an enhanced synthesis of cytochrome P450-derived epoxyeicosatrienoic acids, products that activate Ca^{2+} -activated K^+ channels, have been described (Pfister *et al.*, 1991). Other studies have shown that endothelium-dependent hyperpolarisation is reduced in mesenteric arteries from streptozotocin-induced diabetic rats when compared to age-matched controls (Fukao *et al.*, 1997).

1.4 OXIDANT STRESS AND OTHER DISEASE MEDIATORS

Oxidative stress is the end result of an imbalance between pro-oxidants, including ROS and antioxidant defences, a condition that may result from excess ROS generation or suppressed antioxidant activity.

1.4.1 Reactive Oxygen Species (ROS)

ROS are defined as reactive metabolites of oxygen (Halliwell & Gutteridge, 1989). Superoxide ($O_2^{\cdot-}$), hydroxyl radical (OH^{\cdot}) and hydrogen peroxide (H_2O_2) are referred to as ROS, and can react with biological molecules. The major source of ROS is $O_2^{\cdot-}$ from cell respiration generated by leakage from the mitochondrial electron transport chain and endoplasmic reticulum. Other sources include COX, NADPH oxidation, xanthine oxidase and uncoupled eNOS. The most reactive ROS is OH^{\cdot} , it has an extremely short half life and will attack all molecules in its vicinity. Although somewhat less reactive $O_2^{\cdot-}$, is involved in a number of biological processes and can combine with other molecules to generate compounds with higher reactivity, such as peroxynitrite ($ONOO^{\cdot-}$).

At low concentrations $O_2^{\cdot-}$ acts as an intracellular signalling molecule (Burdon & Rice Evans, 1989). It may also help to regulate vascular reactivity by quenching NO. The reaction of $O_2^{\cdot-}$ with NO produces $ONOO^{\cdot-}$, another potent oxidant that appears to contribute to some of the damaging effects of radicals (Beckman & Koppenoe, 1996); as has been seen in studies of type 2 diabetes patients (Ceriello *et al.*, 2001). $ONOO^{\cdot-}$ can cross cell membranes and is capable of causing oxidative damage to a wide area. In principle, the relative physiological effects of NO can change depending on the availability of $O_2^{\cdot-}$, with the result that impaired vascular reactivity would be a consequence of increasing oxidant levels. Other changes include increased monocyte adhesion to the endothelium, ultimately

leading to the generation of atherosclerotic lesions. At higher concentrations, ROS are able to modify the structure and function of proteins by causing cross-linking or fragmentation.

Since up to 2 % of inspired oxygen may be converted to ROS, it is essential there are mechanisms available to remove them. The endothelium's physiological defence mechanisms for ROS include superoxide dismutase (SOD), catalase and glutathione peroxidase (GPx), glutathione S-transferase and vitamin E (Halliwell & Gutteridge, 1989). SOD catalyses the dismutation of $O_2^{\cdot -}$ to H_2O_2 , which is catalysed to H_2O by GPx or catalase. Antioxidants can be lipophilic, such as vitamin E, or hydrophilic, such as vitamin C, uric acid and plasma proteins. Vitamin E acts as a chain breaking antioxidant that defends the cell against ROS by scavenging radicals present in membranes and lipoproteins as it binds and captures peroxy radicals (chain reaction intermediates) and thus breaks the lipid peroxidation chain reaction. Vitamin C is a water-soluble reducing agent that scavenges $O_2^{\cdot -}$ and OH^{\cdot} , part of its action appears to be the replenishment of vitamin E.

Markers of oxidant stress are indicative of increased levels of ROS. By using end products of lipid peroxidation (Fig. 1.3), a number of methods can be employed to assess oxidative stress in plasma and tissue. These include measurement of thiobarbituric acid reactive substances (TBARS), or more specifically malondialdehyde (MDA; an aldehyde product of lipid peroxidation). However, these simple methods lack specificity and/or sensitivity, or are generally unreliable (Halliwell & Grootveld, 1987). F₂-Isoprostanes are a series of prostaglandin-like compounds which are now established as reliable and sensitive markers of lipid peroxidation (Morrow *et al.*, 1999). They are derived from arachidonic acid largely by an enzyme-independent, free radical catalysed peroxidation (Morrow *et al.*, 1990). They are also likely to be formed *in situ* on phospholipids from which they are subsequently released, pre-formed, presumably by phospholipases (Morrow *et al.*, 1992). F₂-isoprostanes

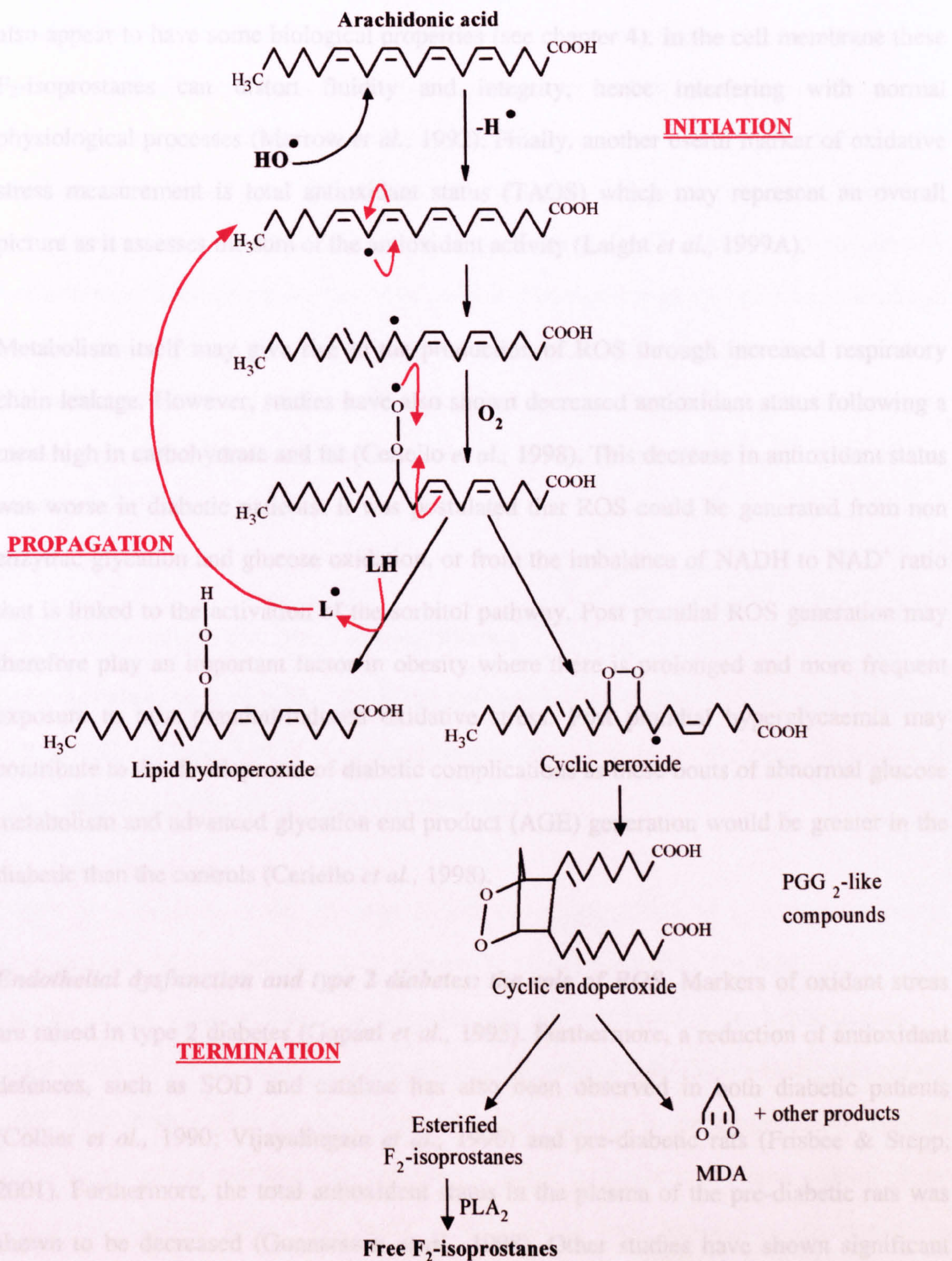


Figure 1.3 The mechanism of lipid peroxidation. There are 3 characteristic phases of lipid peroxidation: Initiation, Propagation and Termination.

also appear to have some biological properties (see chapter 4). In the cell membrane these F₂-isoprostanes can distort fluidity and integrity, hence interfering with normal physiological processes (Morrow *et al.*, 1992). Finally, another useful marker of oxidative stress measurement is total antioxidant status (TAOS) which may represent an overall picture as it assesses the sum of the antioxidant activity (Laight *et al.*, 1999A).

Metabolism itself may give rise to the production of ROS through increased respiratory chain leakage. However, studies have also shown decreased antioxidant status following a meal high in carbohydrate and fat (Ceriello *et al.*, 1998). This decrease in antioxidant status was worse in diabetic patients. It was postulated that ROS could be generated from non enzymic glycation and glucose oxidation, or from the imbalance of NADH to NAD⁺ ratio that is linked to the activation of the sorbitol pathway. Post prandial ROS generation may therefore play an important factor in obesity where there is prolonged and more frequent exposure to post prandial-induced oxidative stress. Post prandial hyperglycaemia may contribute to the development of diabetic complications as these bouts of abnormal glucose metabolism and advanced glycation end product (AGE) generation would be greater in the diabetic than the controls (Ceriello *et al.*, 1998).

Endothelial dysfunction and type 2 diabetes: the role of ROS. Markers of oxidant stress are raised in type 2 diabetes (Gopaul *et al.*, 1995). Furthermore, a reduction of antioxidant defences, such as SOD and catalase has also been observed in both diabetic patients (Collier *et al.*, 1990; Vijayalingam *et al.*, 1996) and pre-diabetic rats (Frisbee & Stepp, 2001). Furthermore, the total antioxidant status in the plasma of the pre-diabetic rats was shown to be decreased (Gunnarsson *et al.*, 1998). Other studies have shown significant improvement in insulin action in type 2 diabetic patients following antioxidant treatment with vitamin E or vitamin C (Paolisso *et al.*, 1993; 1994). It is known that increased oxidant stress causes cell damage (Halliwell, 1994) and even cell death. Increased oxidant stress in

type 2 diabetes may contribute to its complications and more importantly be involved in the primary onset of the disease itself. Oxidant stress can further contribute to the disease progression in type 2 diabetes by altering the balance of mediators involved in vascular regulation. It is known that $O_2^{\cdot-}$ can react with NO causing impaired relaxation, in addition to producing ONOO $^{\cdot-}$, a that radical will elicit endothelial cell damage and prevent any further release of NO from that cell. The decreased availability of endogenous antioxidants in the diabetic patient ultimately means that more free radicals can potentially react with NO and impair vascular function. This impaired vascular function may in turn promote progression of the diabetic disease and enhance its complications (discussed later in this chapter).

1.4.2 Other mediators of type 2 diabetic disease

Other mediators such as leptin, TNF α and the enzyme beta-hydroxysteroid dehydrogenase (11 β -HSD) are described in more detail in this chapter as they have been implicated in type 2 diabetes and the obese Zucker rat pathology.

Leptin is a product of the *ob* gene (Friedman *et al.*, 1991). It is a plasma protein approximately 16 kDa in mass and is secreted from adipose tissue. The leptin receptor is a member of the cytokine family of receptors that are expressed in the hypothalamus, T-lymphocytes and more interestingly on vascular endothelial cells (Sierra-Honigmann *et al.*, 1998). The obese Zucker rat phenotype is due to a mutation in the leptin receptor (OB-R) gene. Leptin acts at hypothalamic receptors to signal increases in the lipid content of the adipocytes, causing satiety and hence a decrease in food intake and an increase in thermogenesis. It is thought to reduce or stop feeding by inhibiting the potent stimulant for feeding, neuropeptide Y (NPY). It has been shown in animals that NPY production can be suppressed by leptin (Stephens *et al.*, 1995). Decreased NPY activates the sympathetic

nervous system and stimulates thermogenesis in brown fat. In contrast, if animals do not have any leptin, the NPY system is completely unrestrained, leading to hyperphagia and hence obesity. Interestingly, obesity in humans appears to be frequently associated with high levels of leptin, so called hyperleptinaemia – suggestive of leptin resistance rather than leptin deficiency in these individuals. This resistance may be due to impaired transport of leptin through the blood brain barrier, or a receptor defect, and/or post receptor failure. Leptin also decreases insulin secretion from β -cells and regulates insulin signalling. Circulating levels of leptin correlate with percentage body fat and fasting insulin concentrations in humans. This makes leptin a possible marker of obesity and the insulin resistance syndrome.

Identification of leptin receptors on the endothelium suggested a vasoactive role for leptin. Leptin has been shown to cause increased blood pressure by increasing sympathetic nervous activity (Dunbar *et al.*, 1997). However, Frühbeck (1999) showed leptin also induced a hypotensive effect in sympathectomised rats by a NO-dependent mechanism. Further investigation showed a direct vasoactive effect by leptin through its functional receptor on the endothelium (Lembo *et al.*, 2000). Leptin also caused vasorelaxation in rat conduit (aorta) and resistance (mesentery) vessels through NO and EDHF -dependent mechanisms respectively. As resistance vessels are thought to have a dominant role in blood pressure homeostasis (Mulvany, 1993), leptin may have a role in the maintenance of blood pressure. In addition, leptin is able to inhibit NPY, which has also been shown to have potent vasoconstrictor properties. Studies have show leptin to increase ROS in human endothelial cells, an effect associated with JNK/SAPK-dependent pathway.

TNF α is a 17-kDa soluble plasma protein secreted from macrophages, monocytes, smooth muscle cells and adipose tissue. It is a proinflammatory cytokine that has been implicated in the pathogenesis of many inflammatory disorders where it acts to enhance the

inflammatory cascade and to activate macrophages to produce ROS. More recently, TNF α has been implicated in obesity, insulin resistance, and type 2 diabetes. Studies have shown an inverse correlation between TNF α levels and both fasting glucose and glucose disposal in normal subjects and those with impaired glucose tolerance (Miyazaki *et al.*, 2003). The study also explained that this correlation is not present in type 2 diabetic subjects and suggested that TNF α may be implicated in the onset of the insulin resistance syndrome but not in the deterioration of peripheral resistance in the later disease. Animal studies in the obese Zucker rat have shown increased plasma TNF α levels, and that neutralization of TNF α *in vivo*, by an infusion of soluble TNF α receptor–IgG protein, significantly increased peripheral insulin-stimulated glucose disposal (Hotamisligil *et al.*, 1993). It is thought that TNF α can potently cause insulin resistance by accelerating lipolysis. This may occur by suppressing the expression of genes that encode proteins that would normally regulate fatty acid uptake or lipogenesis. It is the resultant release of free fatty acids from adipocytes that then block insulin sensitive pathways. Indeed, reduction in both circulating free fatty acid and insulin levels in obese Zucker rats has been reported following TNF α inhibition by infusion of neutralizing TNF antibodies (Morin *et al.*, 1997). TNF α concentrations were decreased in studies using the PPAR γ agonist, troglitazone. In these studies troglitazone increased the number of small adipocytes and decreased the number of large adipocytes in white adipose tissues of obese Zucker rats. The authors suggest this may be an important mechanism by which increased expression levels of TNF α and higher levels of plasma lipids are normalized, leading to alleviation of insulin resistance (Okuno *et al.*, 1998).

11 β -HSD consists of 2 distinct isoenzymes (type 1 and 2) with contrasting functions. 11 β - HSD2 is an exclusive 11 β -dehydrogenase that acts in aldosterone target tissues such as the kidney, colon and sweat glands. It functions as a potent dehydrogenase that rapidly

inactivates cortisol so that aldosterone can selectively access the otherwise nonselective mineralocorticoid receptors. In contrast, 11 β -HSD1 is a predominant 11 β -reductase *in vivo* that acts in many tissues such as the liver, adipose tissue, brain, and lung, to increase local intracellular cortisol concentrations and thereby maintain exposure to its receptors. As 11 β -HSD1 is an enzyme that regenerates cortisol from inactive cortisone, it is a factor capable of producing obesity and type 2 diabetes. The metabolic syndrome X and Cushing's syndrome show similar symptoms including, hypertension, central obesity, glucose intolerance, insulin resistance and dyslipidaemia. However, plasma cortisol is not elevated in syndrome X. However recent evidence has shown increased intracellular cortisol concentrations in adipose tissue in obese humans and Zucker rats (Rask *et al.*, 2001). Interestingly, in these studies also show decreased levels of cortisol in both liver and plasma samples – 11 β -HSD1 is the enzyme responsible for these tissue specific variations in intracellular cortisol concentrations. Recent animal studies have certainly provided greater evidence for the involvement of 11 β -HSD1 in the development of syndrome X. Transgenic mice over-expressing 11 β -HSD1 selectively in adipose tissue developed obesity and showed increased levels of corticosterone in their adipose tissue. Moreover, the mice also exhibited pronounced insulin-resistant diabetes, hyperlipidaemia, and hyperphagia, despite having hyperleptinaemia (Masuzaki *et al.*, 2001). In vascular experiments, it has been reported that 11 β -HSD1 may play an important role in regulating inflammatory responses in the artery wall. 11 β -HSD1 was shown to be expressed in human aortic smooth muscle cells. Treatment of the cells with cytokines, IL-1 β or TNF α caused a time and dose-dependent increase of 11 β -HSD1 mRNA levels, and an increased conversion of cortisone to cortisol (Cai *et al.*, 2001). This is of particular interest as levels of TNF α are elevated in human obesity, and indeed the Zucker rat, and have been associated with insulin resistance. Specific inhibition of 11 β -HSD1 would help to understand aspects of the pathogenesis of syndrome X and may lead to new therapeutic perspectives.

1.5 HYPERGLYCAEMIA

Hyperglycaemia results from ineffective clearance of glucose. Glucose levels are elevated in the plasma, interstitial fluid and in cells where glucose uptake is not insulin-dependent, such as nerve, glomerulus, retina and lens cells. Persistent hyperglycaemia is clearly a major risk factor for type 2 diabetes complications (DCC trial, 1993). The development of endothelial dysfunction in arteries exposed to hyperglycaemia (Valentoric *et al.*, 1985; Tesfamarian *et al.*, 1990) involves increased formation of AGEs, increased glucose flux through the aldose reductase pathway and activation of protein kinase C (PKC) (Nishikawa *et al.*, 2000).

Polyol Pathway. In endothelial cells and cells independent of insulin for glucose uptake, glucose is metabolised by the sorbitol (polyol) pathway. Aldose reductase is an enzyme that converts glucose to sorbitol. Glucose is reduced by aldose reductase, generating NADP^+ , a competitor of glutathione, (Gonen & Dvornik, 1995) and sorbitol, which accumulates intracellularly as it does not easily cross cell membranes (Taylor & Agius, 1998) (Fig. 1.4).

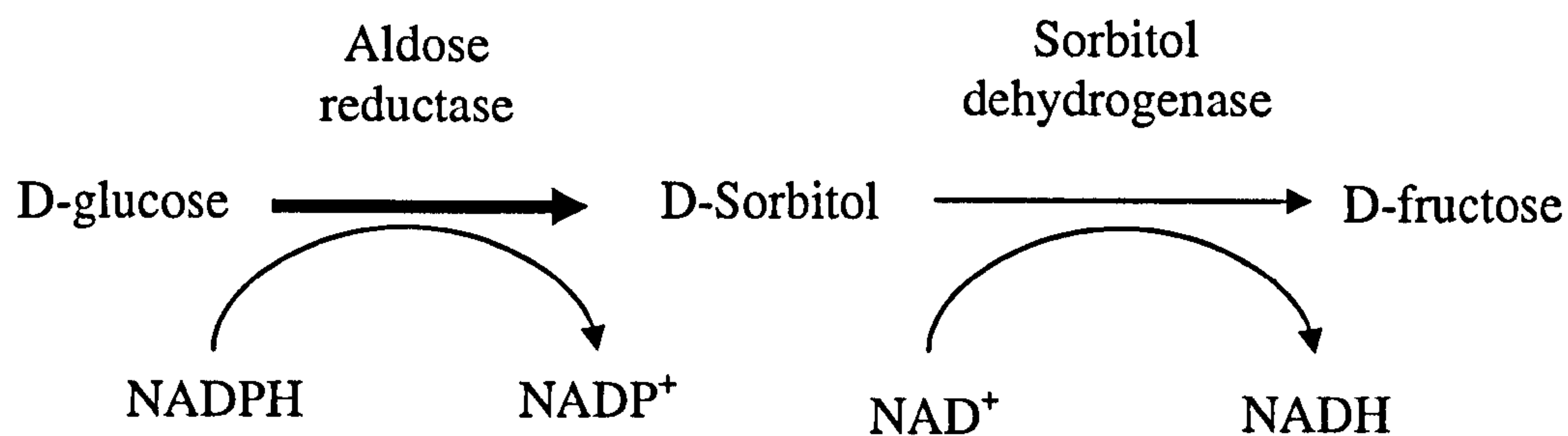


Figure 1.4 Metabolism of glucose via the sorbitol (polyol) pathway.

The polyol pathway is a series of enzymatic reactions in the synthesis of fructose. Normally, aldose reductase has a low affinity for glucose and little glucose substrate enters the polyol pathway. However during hyperglycaemia, this pathway is activated because the K_M of glucose for aldose reductase is high (70 mM). Hyperglycaemia forces intracellular

glucose accumulation, particularly in tissues such as the retina where glucose entry is independent of insulin. The excess glucose is largely metabolized by aldose reductase. It is this increased flux of glucose metabolised through the polyol pathway that is associated with diabetic abnormalities. Hyperglycaemia also leads to *de novo* synthesis of diacylglycerol (DAG), which activates PKC. DAG-PKC is important in the signalling pathways regulating vascular cell permeability and contractility (Koya & King, 1998). It is possible that PKC activation may cause vasoconstriction by increasing endothelin-1 expression in retinal cells (Koya & King, 1998).

AGE. Formation of AGEs occurs by non-enzymatic glycosylation or glycation of glucose, fructose or their phosphates to a protein or lipid. Hence the production of AGE is increased with hyperglycaemia. The reaction occurs between the aldehyde oxygen or keto oxygen and an exposed amino group. The resultant Schiff base then undergoes a number of chemical reactions (rearrangements) leading to the cross linking of proteins. AGEs can cross-link with plasma components and cell surface receptors, which in turn may lead to abnormalities in receptor recognition and enzyme activity. Proteins in contact with the blood are most vulnerable to glycation, and smaller blood vessels may be at particular risk as they have a long replacement time – this can lead to endothelial dysfunction and secondary complications. Investigations *in vitro* have shown glycosylation is proportional to the incubation time and glucose concentration, suggesting increased hyperglycaemia for longer duration will cause more damage associated with diabetic complications. A marker for glycaemic control is glycosylated haemoglobin A1c (HBA1c). Glucose binds irreversibly to haemoglobin and HBA1c is proportional to the concentration of glucose in the blood. Since red blood cells have a lifespan of approximately 90 days, the HBA1c test is a very useful method to assess control of glycaemia in patients. Specific receptors for AGE (RAGE) are also expressed in a number of cell type including monocytes, macrophages endothelial cells, smooth muscle cells and neuronal cells. RAGE expression

is potentiated by hyperglycaemia and TNF α . The binding of AGE to RAGE causes activation of endothelial cells to produce inflammatory mediators including cytokines and adhesion molecules. AGEs accumulate in the extracellular matrix proteins during the physiological process of aging, however earlier and more rapid accumulation of AGE occurs in type 2 diabetic patients. Significant correlation has been shown between AGE deposition and severity of diabetic complications (Wautier & Guillausseau, 2001). Importantly, AGEs have been shown to quench NO both *in vitro* and *in vivo* and thus contribute to endothelial vascular impairment (Bucala *et al.*, 1991). It has been shown that AGE causes microvascular, retinal and glomerular and nerve lesion that can be prevented with aminoguanidine (inhibitor of AGE formation). In addition, other studies have shown that recombinant RAGE prevented hyperpermeability and vascular lesion. Glycation of oxidised lipids and lipoproteins further increases their atherogenicity and may, at least in part, explain why diabetics have increased risk for cardiovascular disease.

1.5.1 Diabetic Complications

The contributory risk factors to the development of diabetic complications include – age, poor glycaemic control, duration of diabetes, and abnormal lipid metabolism. Current evidence suggests that each late complication of diabetes arises following a sequence of progressive changes to an individual organ or system, and hyperglycaemia is the common insult initiating the early complication. The four major pathways of glucose metabolism involved in the development of diabetic complications include: 1. Increased polyol pathway activity leading to sorbitol and fructose accumulation, NAD(P)H-redox imbalances, and changes in signal transduction; 2. Non-enzymatic glycation of proteins yielding AGEs; 3. Activation of PKC thereby initiating a cascade of stress responses, and 4) Increased hexosamine pathway flux (Fig. 1.5). In the diabetic state increases in polyol pathway activity, AGE accumulation, PKC activity, and hexosamine flux trigger a feed-forward

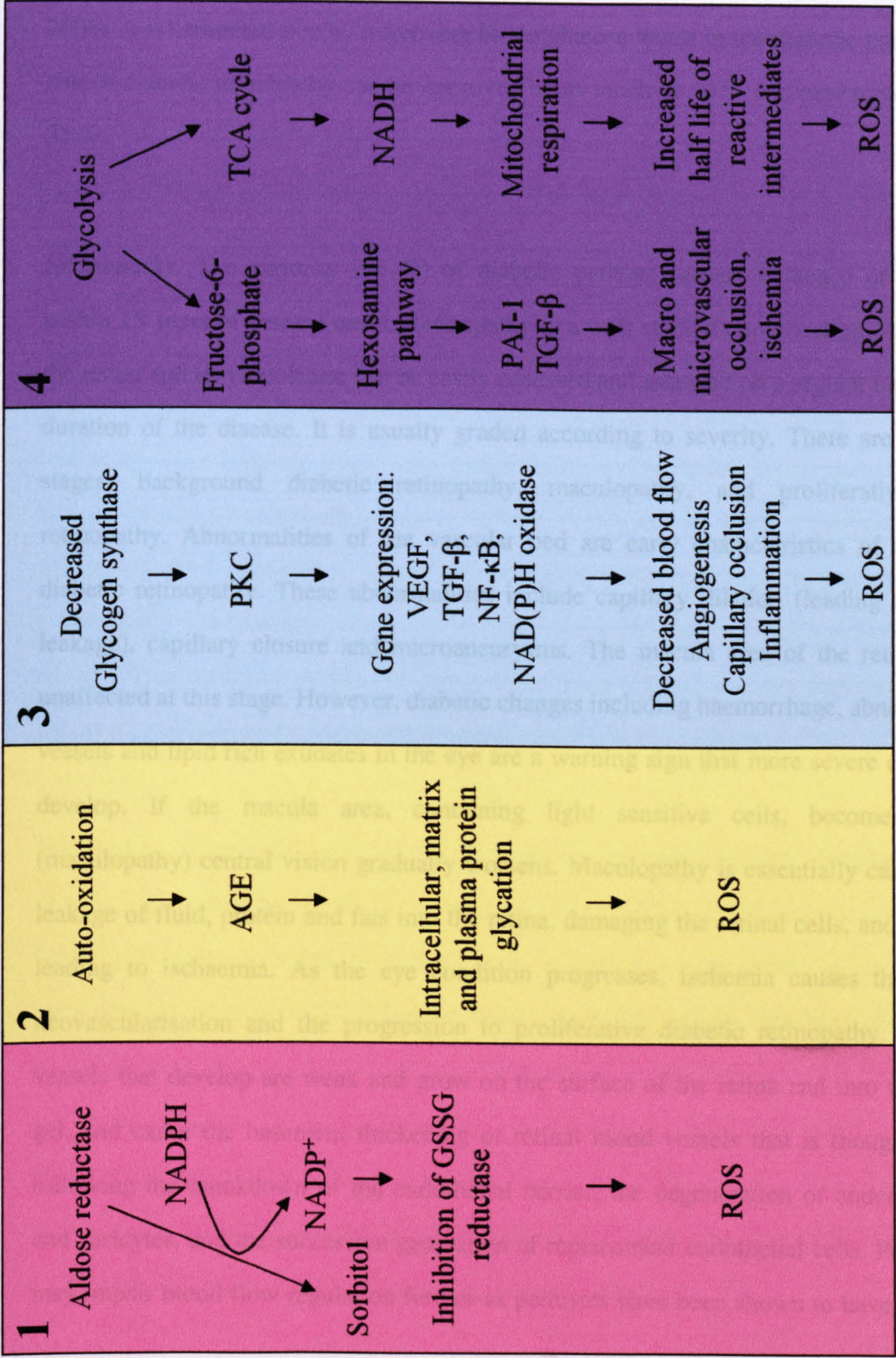


Figure 1.5. The four major pathways of glucose metabolism involved in the development of diabetic complications and the production of ROS include 1) Increased polyol pathway activity 2) Nonenzymatic glycation of proteins 3) Activation of PKC and 4) Increased hexosamine pathway flux. Illustration adapted from Feldman, *J. Clin. Invest.*, 2003.

system of for increased $O_2^{\cdot -}$ accumulation and progressive cellular dysfunction (Feldman, 2003). It is estimated that by improving blood glucose levels in the diabetic patient that the risk of diabetic retinopathy can be improved by as much as 50 % and nephropathy damage 33 %.

Retinopathy. The majority (90 %) of diabetic patients present evidence of retinopathy within 15 years of disease onset. Retinopathy is a well studied complication of diabetes as the retina and its vasculature can be easily accessed and assessed on a regular basis over the duration of the disease. It is usually graded according to severity. There are three main stages; background diabetic retinopathy, maculopathy, and proliferative diabetic retinopathy. Abnormalities of the vascular bed are early characteristics of background diabetic retinopathy. These abnormalities include capillary dilation (leading to capillary leakage), capillary closure and microaneurysms. The macula area of the retina remains unaffected at this stage. However, diabetic changes including haemorrhage, abnormal blood vessels and lipid rich exudates in the eye are a warning sign that more severe disease may develop. If the macula area, containing light sensitive cells, becomes involved (maculopathy) central vision gradually worsens. Maculopathy is essentially caused by the leakage of fluid, protein and fats into the retina, damaging the retinal cells, and eventually leading to ischaemia. As the eye condition progresses, ischemia causes the need for neovascularisation and the progression to proliferative diabetic retinopathy. The blood vessels that develop are weak and grow on the surface of the retina and into the vitreous gel, and cause the basement thickening of retinal blood vessels that is thought to occur following the breakdown of the endothelial barrier, the degeneration of endothelial cells and pericytes, and the successive generation of replacement endothelial cells. Pericyte loss may impair blood flow regulation further as pericytes have been shown to have contractile

properties. This thickening of the retinal blood vessels eventually forms fibrous tissue which can distort the retina, leading to retinal detachment.

The probable factors proposed in the pathogenesis of retinopathy are increased polyol pathway activity, non enzymic glycation of structural proteins and enhanced mechanical force delivered to the vessel by rigid red blood cells. The resultant changes in endothelial cells include decreased NO levels, and enhanced activity of the vasoconstrictors endothelin-1 and angiotensin II which pushes the vessels towards constriction and retinal ischemia. In addition, glycation of haemoglobin can reduce the oxygen capacity of red blood cells and lead to hypoxia. Hypoxia alters vessel permeability and facilitates the release of angiogenic factors. Vascular endothelial growth factors (VEGFs) and their receptors VEGFR-1, VEGFR-2, and VEGFR-3, control pathological angiogenesis and increase vascular permeability and have been shown to be over expressed in diabetic retinopathy (Witmer *et al.*, 2003). In addition, AGEs binding to their receptors will activate endothelial cells to produce cytokines, and express adhesion molecules and tissue factor.

Neuropathy. It has been estimated that 20 to 40 % of diabetics develop neuropathy, the risk of which increases by 15 % for every 1 % rise in HbA1c. Diabetic neuropathy is a progressive disease caused by deterioration of nerves which results in peripheral and autonomic nerve dysfunction. Nerve damage caused by diabetes generally occurs over a period of years and may lead to problems with internal organs including the digestive tract and sexual organs. Diabetic neuropathy can be divided into three groups: 1. diffuse (peripheral) neuropathy (affects the legs, feet, arms, hands). 2. diffuse (autonomic) neuropathy (affects the heart, digestive system, sexual organs, urinary tract, sweat glands). 3. focal neuropathy (eyes, probably due to pressure damage).

It is the peripheral nervous system that is most commonly affected and sensory nerve fibres are usually the first affected. They show a reduction in the conduction velocity, together with reduced amplitude and increased temporal dispersion of the action potential. Neurophysiological studies have also shown reduced motor and sensory nerve conduction in ischemic nerves. Nerve biopsies obtained from diabetic patients have shown axonal degeneration and regeneration, demyelination and remyelination, and capillary closure. In addition, the extent of damage was correlated to severity of disease. These impaired neural functions and loss of neurotrophic support, can eventually mediate apoptosis of neurons, Schwann cells, and the glial cells of the peripheral nervous system.

The precise mechanisms causing nerve damage still needs to be established. Possible causes include osmotic swelling secondary to intracellular sorbitol accumulation; this is known to cause functional and morphological alterations in the diabetic nerve, also myoinositol depletion leading to reduced Na^+ K^+ ATPase activity, and increased intracellular Na^+ levels that in turn slow nerve conduction velocity. It is known that RAGEs can modulate cell activation, growth related mediators and cell proliferation. Therefore in neuropathic complications AGE contributes to reduced nerve perfusion, and endoneural hypoxia changes in nerve microvascular including basement membrane thickening, pericyte degeneration and endothelial cell hyperplasia. Studies into nerve growth factor (NGF) have shown it was decreased in diabetic subjects. However, further investigations into its potential as a causative factor of neuropathy are disappointing, as the results from recent clinical trials failed to prove efficacy (Apfel, 2002). More recently, the Hedgehog Family (Sonic, Desert and Indian) of proteins which play a key role in the formation, maintenance and repair of a number of tissues, including the nervous system and cartilage, was shown to have reduced mRNA expression in the peripheral nerve of maturing diabetic rats. These studies showed that treatment with a sonic hedgehog-IgG fusion protein fully restored motor- and sensory-nerve conduction velocities and maintained the axonal calibre of large

myelinated fibres (Calcutt *et al.*, 2003). Other studies in animal have shown that antioxidant treatment can prevent or reverse the hyperglycaemia-induced nerve dysfunction, possibly via correction of nutrient blood flow (van Dam, 2002).

Nephropathy. The prevalence of diabetic nephropathy is 5 – 60 % of type 2 diabetics depending on ethnic origin (Caucasians 5 – 10 %, African Americans 10 – 20 %, Pima Indians 60 %). Nephropathy is defined by persistent albuminuria, a declining glomerular filtration rate (GFR) and rising blood pressure. The earliest clinical evidence of nephropathy is the appearance of low levels of albumin (microalbuminuria) in the urine, referred to as incipient nephropathy. Once overt nephropathy occurs, without specific interventions, the GFR gradually falls. Albuminuria is due to glomerular capillary damage and reflects generalised damage to microcirculation and large vessels. Microalbuminuria is due to increased permeability of glomerular capillaries, probably due to raised pressure. Hypertension, also a complication of diabetes, accelerates the rates at which albuminuria increases and GFR declines. Proteinuria itself can lead to low albumin levels and peripheral oedema.

The proposed mechanisms involved in the progression of nephropathy again involve increased activity of the polyol pathway. This increased polyol pathway activity impairs myoinositol metabolism in the tissues in which intracellular glucose concentrations are not regulated by insulin. Myoinositol is required to maintain synthesis of phosphatidylinositol, whose rapid turnover regulates a major component of tissue Na⁺ K⁺ ATPase activity. Studies have shown that myoinositol supplementation and aldose reductase therapy improve glomerular function and increase nerve conduction velocity (Goldfarb *et al.*, 1991). Another mechanism of damage is glycation of albumin, which has been shown to modulate signal transduction and induce alterations in renal glomerular cells that contribute to the development of diabetic nephropathy.

Macrovascular disease. Diseases of the large arteries are responsible for over 50 % of diabetic deaths. Peripheral vascular disease is the name of the group of problems caused by inflammation and structural damage in the blood vessels. One of the major diseases in this group is atherosclerosis. Atherosclerosis is caused by fatty build up inside the artery walls over time which can lead to occlusion and restriction of blood flow. In early atherosclerosis, monocytes adhere to the endothelium and migrate into the intima in a three stage process (rolling, activation and transmigration). This process is mediated by selectins and adhesion molecules on the endothelium, such as ICAM-1 and VCAM-1. The selectins and adhesion molecules first cause the monocytes to slow, then their adhesion to the endothelium, followed by their migration through the intercellular junctions into the sub-endothelial space. Hyperglycaemia has been shown to increase monocyte binding in human aortic endothelial cells (Kim *et al.*, 1994). More recently, insulin resistance and adiposity was shown to correlate with acute-phase reaction and soluble cell adhesion molecules in type 2 diabetes (Leinonen *et al.*, 2003). The monocytes differentiate into macrophages and accumulate lipid to form foam cells. The lipid laden foam cells form the characteristic fatty streaks of early the atherosclerotic lesion. Production of growth factors and cytokines by foam cells then initiate the migration of smooth muscle cells from media into the intima and their proliferation. The advanced lesion consists of an extracellular lipid core and a thick fibrous structure in the lumen, this type of lesion narrows the artery. Finally the complicated plaque can fissure and rupture to cause myocardial infarction or stroke.

The vascular imbalance of vasoregulators such as NO and O₂[•] contribute to the disease progression from diabetes to atherosclerosis. NO has potent vasoprotective effects including inhibition of vascular smooth muscle cell proliferation, lipid peroxidation, and monocyte adhesion to endothelial cells – all of which are important in the prevention of

atherosclerotic disease progression. In addition, NO inhibits platelet adhesion and thrombogenesis, an important factor in the prevention of myocardial infarction and stroke in the later stage of atherosclerosis. The decreased NO availability in frank diabetes may also contribute to hypertension, especially in individuals with atherosclerosis arterial narrowing. Finally, NO counterbalances other vasoconstrictors such as endothelin, angiotensin II and more relevant to this thesis, $O_2^{\cdot-}$.

1.6 ANIMAL MODELS OF TYPE 2 DIABETES

The term model refers to a reproduction of a system on a different scale. The advantage of an animal model of disease is analysis of metabolic or physiological parameters not or not easily accessible in humans, including tissue biopsy (Renold *et al.*, 1988). There are many different types of animal models available that provide routes of investigation into diabetes. Some of the animal models available include; the immune related BB rat and NOD mouse; the NIDDM-IDD transition *db/db* mouse; the low insulin secretion NON mouse; the nutrient-dependent sand rat or spiny mouse; the genetically selected Goto-Kakizaki (GK) rat, and the obesity related *ob/ob* mouse, Otsuka-Long-Evans Tokushima Fatty Rat (OLETF) and *fa/fa* rats. In addition, diabetes can be chemically induced in normal rats, such as the Wistar rat, by using drugs that are toxic to the pancreatic β -cells, such as alloxan and streptozotocin (STZ). None of these models are identical to the human syndrome, but all have mechanisms that cause type 2 diabetic and type 1-like disease. The mechanism and progression of disease is worth investigation as its presence in one system helps the understanding of another system. This is especially fitting in understanding a polygenic disease such as type 2 diabetes.

1.6.1 The obese Zucker “fatty” rat (*fa/fa*)

The Zucker “fatty” rat (*fa/fa*) (Fig. 1.6) is model of obesity, insulin resistance and pre-type 2 diabetes, resulting from a cross between Sherman and Merck rats (Zucker and Zucker, 1961). The homozygous phenotype Zucker (*fa/fa*) rat exhibits obesity is due to a single missense mutation at codon 269 (glutamine to proline) of the leptin receptor (OB-R) gene. This reduces the expression of the leptin receptor on the cell surface leading to marked intracellular retention, decreased leptin binding and diminished signal transduction. The non-obese genotypes with the (*fa*) gene are appropriate age matched controls for the obese (*fa/fa*) Zucker rat, as levels of glucose metabolism in fat cells are the same (Cleary & Phillips, 1996).

The obese Zucker rat (*fa/fa*) has several endocrine abnormalities that accompany its obesity including hyperleptinaemia, hyperphagy, hyperinsulinaemia, insulin-resistance, hyperglycaemia and hypercholestromia. However it does not appear to develop atherosclerosis spontaneously. Other endocrine disorders include depressed pituitary growth hormone secretion, hypothyroidism, oversecretion of glucagon, abnormal regulation of the thermogenic process, and reproductive dysfunction. The marked insulin resistance concomitant to hyperinsulinaemia may account for the reported normoglycaemia of these animals. It is interesting that administration of corticosterone to normal rodents caused hyperphagia, hyperinsulinaemia, increased energy efficiency, increased lipid, but decreased protein deposition. This demonstrates the importance of leptin and the hypothalamic-pituitary axis in this disease model and its role in type 2 diabetes. The prevalence of psychosocial stress factors and their association with increased body fat (mentioned earlier (Bjorntorp, 1991)) was studied in lean and obese rats using fluoxetine (a serotonin reuptake inhibitor). Fluoxetine administration caused a reduction in NPY levels and NPY secretion

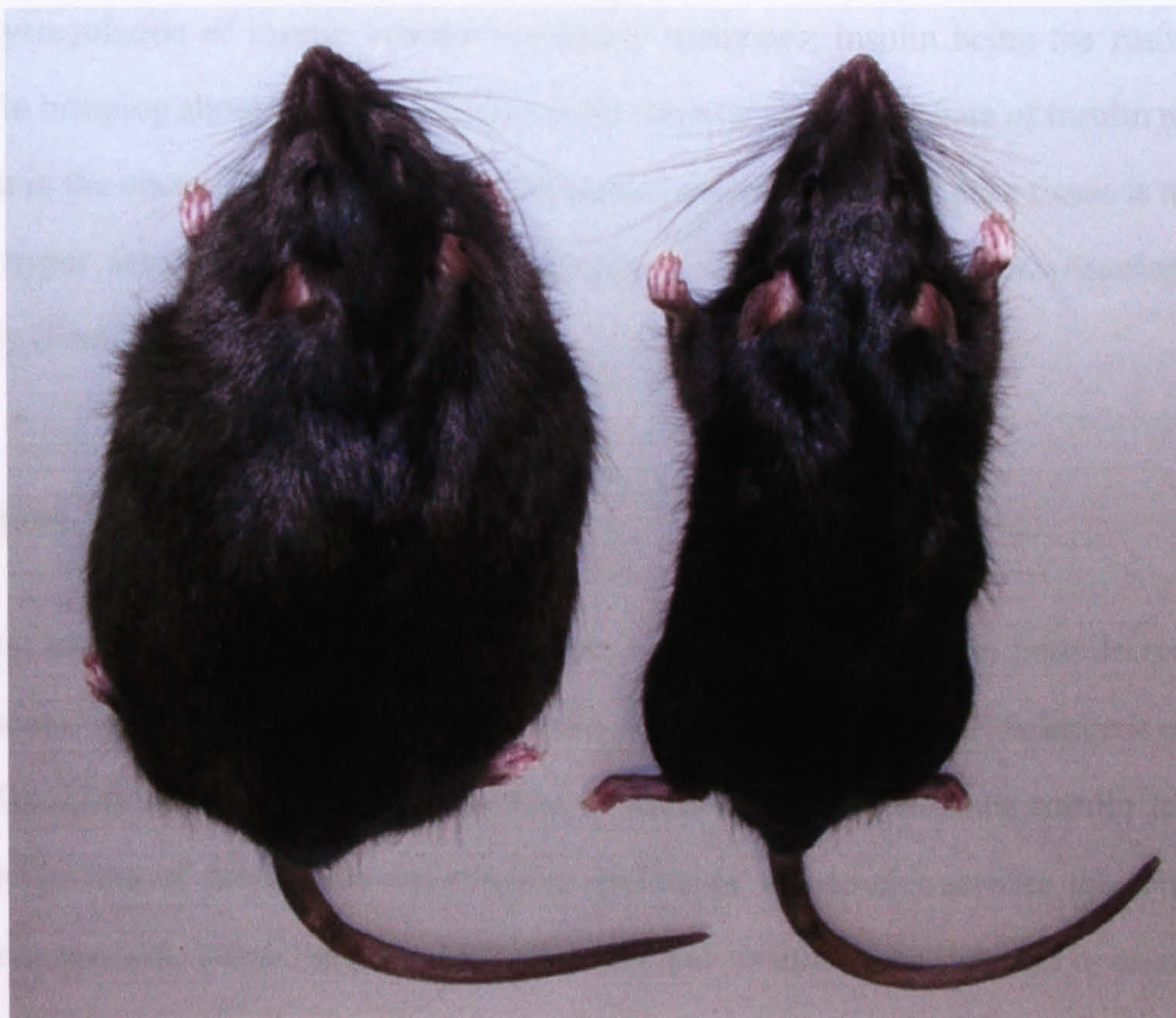


Figure 1.6 Comparison of the size of the lean and obese Zucker rat littermates at 13 weeks-old

in the hypothalamus, resulting in hypophagia and weight loss (Dryden *et al.*, 1996). The pathological progression in the Zucker rat has been proposed as a central nervous system (CNS) and autonomic nervous system (ANS) disorder that results in insulin oversecretion and dysregulation of insulin counter regulatory hormones; insulin being the main driving force in bringing about insulin resistance in the skeletal muscle. A state of insulin resistance is seen in the obese Zucker liver skeletal muscles, whereas white adipose tissue is normal or even hyper sensitive to the effects of insulin thus contributing to the development of obesity (Penicaud *et al.*, 1987).

1.7 AIMS AND OBJECTIVES

Oxidant stress has been associated with type 2 diabetes. For example, both decreased NO and increased in $O_2^{\cdot-}$ plasma levels have been reported. If the NO/ $O_2^{\cdot-}$ balance is perturbed vascular dysfunction can result. Similarly, oxidant stress may enhance insulin resistance. The initial aim of the studies described in this thesis was to characterise the relationship between oxidant stress, endothelial function and insulin resistance. To assess these relationships, new methods to measure endothelial function in animal disease models needed to be developed. Firstly, the *in situ* perfused hindquarters would allow direct measurement endothelial function without autonomic nervous control and blood. In addition to it being a large vascular area which is insulin sensitive. Secondly, was to develop an *in vivo* model to assess endothelial function with autonomic control using Doppler ear blood flow measurements. These models could provide data that could be extrapolated to isolate peripheral effects in particular endothelium-dependent effects of the pharmacological interventions *in vivo*. Endothelial function and oxidant stress could then to be investigated in the obese Zucker rat – a model of insulin resistance. This model provided information on the presence of endothelial dysfunction and oxidant stress in a pre-diabetic model. This being of interest, in that it is known that oxidants contribute to the

complications associated with type 2 diabetes, but their causative role is less clearly understood. Similarly, endothelial dysfunction is certainly a symptom of type 2 diabetic disease but it having a causative role in early disease is not clear.

Once oxidant stress, endothelial dysfunction and insulin resistance were understood to exist in the obese Zucker rat, the next aim was to test the hypothesis that increasing oxidant stress contributes to an altered vascular function and impaired insulin regulation. Thus, oxidant stress contributes to the disease progression in the obese Zucker rat. This aim was tested by increasing the oxidant stress levels by chronic administration of pro-oxidants to the rats. The obese Zucker rat already exhibited increased plasma levels of the oxidant stress marker 8-epi PGF_{2α}. In addition, fructose a natural dietary ingredient could additively increase production of oxidant stress by another mechanism, pushing this pre-diabetic syndrome to a later stage of diabetic disease. The deterioration of endothelial function and insulin resistance would provide evidence for the capability of oxidant stress to worsen type 2 diabetic disease. Finally, further evidence for the role of oxidant stress and endothelial dysfunction in pushing the insulin resistant syndrome to frank diabetes was to test the notion that a decrease in oxidant stress with antioxidants, would correspond to enhanced endothelial function.

Chapter 2

General Methods and Materials

2.1 MATERIALS

Solvents were obtained from Rathburn Chemicals Ltd, (Walkerburn, UK). All other chemicals were obtained from Sigma Chemical Co. (Poole, Dorset, UK) unless otherwise stated below.

Thiopentone sodium (Intraval sodium) and sodium pentobarbitone (National Veterinary Supplies, Stoke on Trent, UK); CaCl_2 (BDH); 8-epi-PGF₂ α (Cayman chemical company, Ann Arbor Michigan, USA); 8-epi-PGF₂ α radioimmunoassay (RIA) (Oxford biomedical Research, Oxford, MI, USA); Evans Blue (Boehringer Ingelheim, Heidelberg, Germany); Fructose (fructofin C, Forum Products, Surrey, UK); Human recombinant insulin and NADPH (Boehringer Mannheim Biochemica, Germany); Insulin radioimmunoassay (RIA) (Linco Research Inc. St. Charles, Missouri, USA).

2.2 GENERAL METHODS

2.2.1 Perfusion of the rat hindquarters *in situ*

Male Wistar rats (250-350 g) were anaesthetised with pentobarbitone sodium (60 mg kg⁻¹ i.p.) and heparin (200 U kg⁻¹) administered via the tail vein. A midline laparotomy was performed and the intestines displaced to reveal the abdominal aorta and vena cava. The aorta was ligated and then cannulated for perfusate inflow at the bifurcation point of the common iliac arteries. The vena cava was cannulated at the same point to allow perfusate outflow (effluent). The abdominal incision was then closed with clips and the animal exsanguinated. The temperature of the hindquarters was monitored via a rectal probe and maintained at 37°C by a homeothermic blanket. The hindquarters were perfused in an open circuit with physiological salt solution (PSS) warmed to 37°C and gassed with 95 % O₂ and 5 % CO₂. The composition of the PSS in (mM): NaCl 133, KCl 4.7, NaH₂PO₄ 1.35, NaHCO₃ 16.3, MgSO₄ 0.61, CaCl₂ 2.52, and d-glucose 7.8. PSS was perfused at a constant

flow of 7 mL min⁻¹. The changes in perfusion pressure, reflecting changes in vascular resistance, were measured by a pressure transducer and continuously recorded using a MacLab system (Hastings, Sussex, U.K.). A stabilisation time of 20 min was allowed before proceeding with experimental protocols. The delivery of drugs was via an injection port proximal to the peristaltic pump (Fig. 2.1). The initial validation studies performed with this method are included in chapter 3.

In agonist stimulated endothelial function studies, Phe eliciting approximately 85 % of the maximal response (Zucker obese and lean: 100 nmol min⁻¹) was infused at t=20 min to provide sub-maximal pre-constriction. After a stabilisation of induced vascular tone, bolus doses of ACh (0.001-30 nmol; 10-30 µl) or sodium nitroprusside (SNP) (0.001-100 nmol; 10-30 µl) were injected to construct vasodilator dose response curves.

In separate experiments, the effect of N^G-nitro-L-arginine methyl ester (L-NAME) on an ACh (0.001-30 nmol) dose response curve was assessed in hindquarters preparations pre-constricted with Phe (100 nmol) at t=20 min. L-NAME (2.1 µmol equivalent to 300 µM) (Rees *et al.*, 1990) was added to the PSS of the treated preparations at t=0 min. After a stabilisation of induced vascular tone, bolus ACh was administered.

In experiments assessing the acute action of insulin, hindquarters were exposed to insulin (100 nM) or its vehicle BSA (0.1 %) for 20 min. Then the hindquarters were pre-constricted with Phe (300 nmol). After the constriction was stabilised, endothelium-dependent vasodilation to ACh (0.001-30 nmol), and endothelium-independent vasodilation to SNP (SNP, 0.01-100 nmol), were assessed in separate preparations. In other preparations, constriction to Phe (0.001-10 nmol) was assessed following a 20 min pre-treatment with human insulin (100 nM) or its vehicle in the presence or absence of L-NAME (2.1 µmol). L-NAME was added 10 min prior to constriction with Phe.

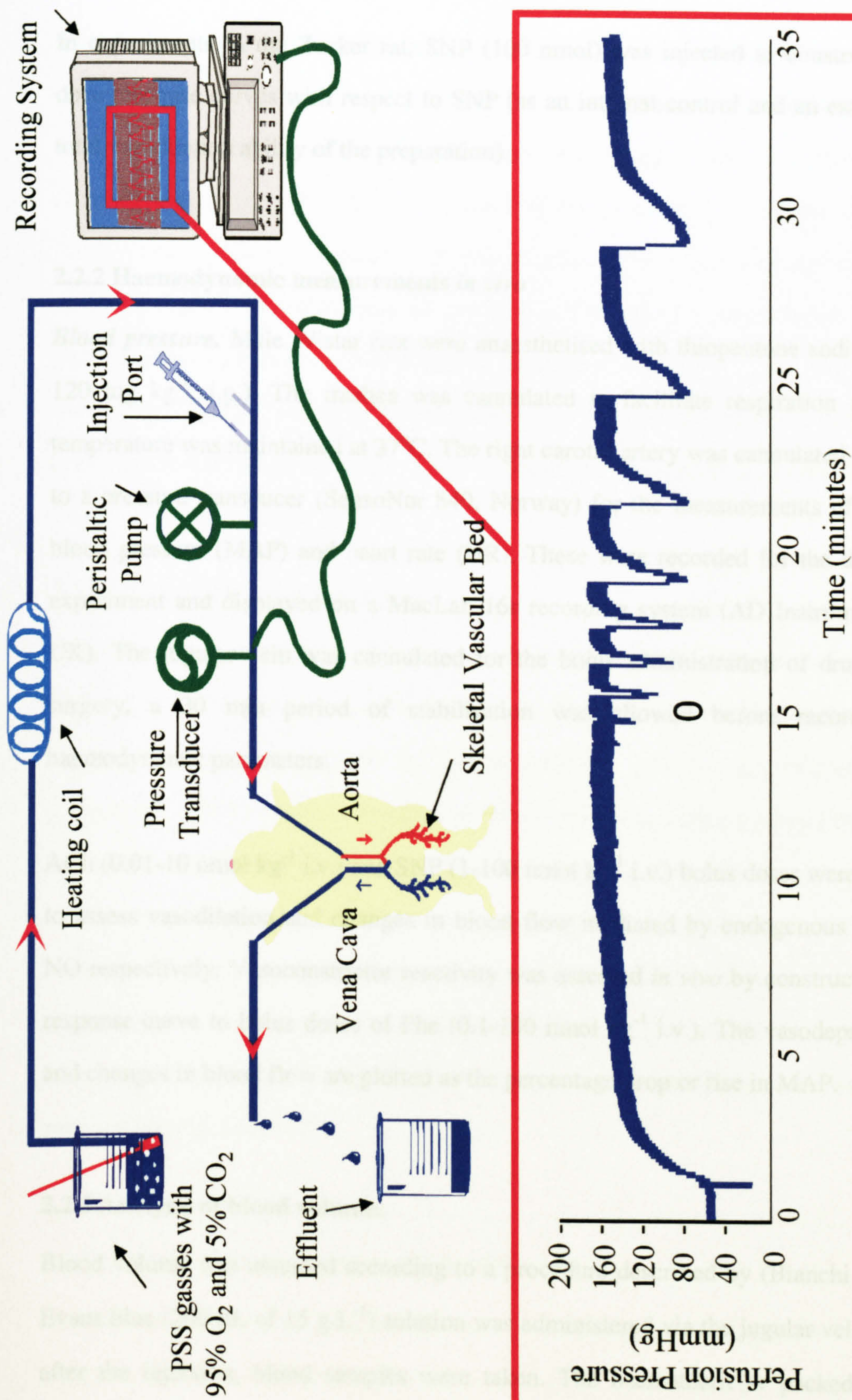


Figure 2.1 Experimental setup and typical trace for measurement of pressure in the rat perfused hindquarters *in situ*. The trace shows changes in perfusion pressure to bolus dose acetylcholine in a phenylephrine pre-constricted preparation. The computer acquisition rate was 200 samples per second.

In experiments in the Zucker rat, SNP (100 nmol) was injected to construct vasodilator dose response curves with respect to SNP (as an internal control and an estimation of the total vasodilation ability of the preparation).

2.2.2 Haemodynamic measurements *in vivo*

Blood pressure. Male Wistar rats were anaesthetised with thiopentone sodium (Intraval[®], 120 mg kg⁻¹, i.p.). The trachea was cannulated to facilitate respiration and the rectal temperature was maintained at 37°C. The right carotid artery was cannulated and connected to a pressure transducer (Sensonor 840, Norway) for the measurements of mean arterial blood pressure (MAP) and heart rate (HR). These were recorded for the duration of the experiment and displayed on a MacLab 16s recording system (AD Instruments, London, UK). The jugular vein was cannulated for the bolus administration of drugs. Following surgery, a 30 min period of stabilisation was allowed before recording baseline haemodynamic parameters.

ACh (0.01-10 nmol kg⁻¹ i.v.) and SNP (1-100 nmol kg⁻¹ i.v.) bolus doses were administered to assess vasodilation and changes in blood flow mediated by endogenous or exogenous NO respectively. Vasoconstrictor reactivity was assessed *in vivo* by construction of a dose response curve to bolus doses of Phe (0.1-100 nmol kg⁻¹ i.v.). The vasodepressor activity and changes in blood flow are plotted as the percentage drop or rise in MAP.

2.2.3 Analysis of blood volume.

Blood volume was assessed according to a procedure described by (Bianchi *et al.*, 1981). Evans blue (200 µL of 15 g L⁻¹) solution was administered via the jugular vein. At t=5 min after the injection, blood samples were taken. The haematocrit or packed cell volume

(PCV) was determined in duplicate, and blood was centrifuged at 15,000 rpm for 5 min to derive the plasma. Evans Blue ($0.5\text{--}32\text{ mg L}^{-1}$) standard curve was constructed and distilled water diluted plasma samples (1:10) were read at 621 nm in a spectrophotometer (Shimadzu UV-160). The plasma volume was calculated by the formula: Plasma volume (cm^3) = (Absorbance of standard / Absorbance of sample) \times 10, where the standard is 3 mg of Evans blue in 10 cm^3 plasma diluted (1:10). The use of a single blood sample for the determination of plasma is well accepted (Bianchi *et al.*, 1981). Total blood volume was determined from the equation: Blood volume (cm^3) = Plasma volume \times $100/100 - (\text{PCV} \times 0.95)$, where 0.95 is the correction factor to obtain true cell percentage (total body PCV).

2.2.4 Glucose tolerance test (g.t.t.)

Rats deprived of food for 16-20 hours were anaesthetised with thiopentone sodium (120 mg kg^{-1} , i.p.). The trachea was cannulated to facilitate respiration. The carotid artery and jugular vein were cannulated for blood sampling and the administration of drugs respectively. After a stabilisation time of 30 min following surgery a basal sample of blood ($500\text{ }\mu\text{L}$) was collected in EDTA (10 mg ml^{-1} blood) to determine basal levels of glucose and insulin. The volume of blood lost in sampling was replaced with an equal volume of saline. A D-glucose solution (0.5 g kg^{-1}) was administered as a bolus dose over 10 sec. Blood samples were then collected at times 1, 3, 6, 12, 24 min post glucose bolus. The blood was centrifuged at 15,000 rpm for 3 min at 4°C and the plasma frozen in liquid nitrogen and stored at -70°C .

2.2.5 Collection of plasma for analysis of 8-epi-PGF_{2 α}

Rats deprived of food for 16-20 hours were anaesthetised with pentobarbitone sodium (60 mg kg^{-1} , i.p.) and exsanguinated by the removal of blood through the abdominal aorta.

Blood was collected into a syringe containing sodium citrate (3.8 % w.v⁻¹), indomethacin (15 µM) and butylated hydroxytoluene (BHT) (20 µM). This was then centrifuged at 15,000 rpm for 3 min at 4°C to derive plasma. The plasma was further treated with BHT (20 µM) and immediately frozen in liquid nitrogen and stored at -70°C.

2.2.6 Extraction of 8-epi-PGF_{2α} from plasma

Plasma was mixed with a Folch solution (CHCl₃: MeOH, 2:1) containing MgCl (0.43 %). The samples were then centrifuged at 1000 rpm for 3 min to separate the water, protein, and organic layers. The organic layer containing the isoprostanes was evaporated to dryness under N₂ and reconstituted in MeOH. The samples were incubated for 30 min at 37°C with KOH (15 %) to isolate the total isoprostane content from the cell membranes into solution. The sample solution was later neutralised with water (pH 3).

The samples were applied to a C₁₈ sep-pak cartridge that had been preconditioned with MeOH then water (pH 3). After sample addition, the column was washed with water (pH 3) and heptane and eluted with ethyl acetate and heptane (1:1). Sodium sulphate was added to the eluent to remove excess water. A Silica sep-pak column was preconditioned with MeOH then ethyl acetate, before the eluate from the C₁₈ Sep-pak was loaded. The column was washed with ethyl acetate: methanol (1:1), and the final eluate was evaporated under N₂ then reconstituted in the immunoassay dilution buffer (supplied by Oxford biomed).

2.2.7 Measurement of 8-epi-PGF_{2α} by Enzyme Immunoassay and GCMS

The EIA kit (EA 84) was purchased from Oxford biomedical Research. The assay is based on the competition between free 8-epi-PGF_{2α}, in the standards or samples, competing with 8-epi-PGF_{2α} linked to horseradish peroxidase (HRP) for a limited number of sites on a 8-

epi-PGF_{2α}-specific polyclonal antibody coated on a microtitre plate. The HRP enzyme activity results in colour development proportional to the amount of 8-epi-PGF_{2α} linked to HRP that is bound and inversely proportional to the amount of free 8-epi-PGF_{2α} in the samples or standards. The assay was carried out as described in the protocol except that plates were washed manually. GCMS samples were analysed in collaboration with Dr N. Gopaul and Ms S. Dhir, in a method essentially described by Gopaul (2000).

2.2.8 Effect of 8-epi-PGF_{2α} in rat perfused hindquarters.

Following stabilisation, the Wistar rat hindquarters were perfused with PSS with or without L-NAME (2.1 μmol) for 20 min before and during the administration of cumulative concentrations of 8-epi-PGF_{2α} (0.1-300 nmol)

2.2.9 Effect of vasodilators and vasoconstrictors in rat aortic rings.

Rats were anaesthetised with pentobarbitone sodium (i.p.) and exsanguinated by the removal of blood through the abdominal aorta. The thoracic aorta was removed and cleaned of adhering connective tissue and cut into 3 mm rings. The rings were mounted at random under a 2 g resting tension in organ baths filled with a PSS at 37°C (see above) and gassed with 95 % O₂ and 5 % CO₂. The aortic rings were allowed to equilibrate for 1 hour, during which the PSS was changed at 15 min intervals. Tension was recorded from Grass FT03C isometric force transducers onto a MacLab recording system.

Experimental protocols. Cumulative contraction curves to Phe (0.0001-10 μM) or 8-epi-PGF_{2α} (0.01-30 mM) were obtained depending on the experiment. ACh (10 μM) was added to assess the integrity of the endothelium. A Phe constrictor tone was induced, followed by

a cumulative relaxation curve to ACh (0.0001-10 μ M). SNP (100 μ M) was added at the end of the experiment to assess the vascular reactivity.

In experiments assessing the acute action of insulin, rings were randomly exposed to insulin (100 nM), or its vehicle BSA (0.1 %), for 20 min. Then they were pre-contracted with noradrenaline (100 nM), a concentration that elicits approximately 90 % of the maximal response. After the pre-contraction stabilised endothelium-dependent vasorelaxation to ACh (1-1000 nM), and endothelium-independent vasorelaxation to SNP (SNP, 1-300 nM), were assessed in separate preparations. In other preparations, contraction to Phe (0.001-10 μ M) was assessed following a 20 min pre-treatment with human insulin (100 nM), or its vehicle, in the presence or absence of L-NAME (300 μ M). L-NAME was added 10 min prior to contraction with Phe. Insulin (100 nM), or its vehicle, was present throughout the experiment.

2.2.10 Antioxidant and Pro-oxidant treatment regimes

For antioxidant studies, male 9 week-old Zucker rats (Harlan, Blackthorn, Bicester, UK) were maintained for 4 weeks on either a standard chow diet (Special Diet Services, Witham, Essex, UK) or a chow enriched with probucol or Vitamin E ((\pm)- α -tocopherol acetate) – each antioxidant was in the chow at 0.5 % ww⁻¹. Alternatively, the intracellular superoxide scavenger tiron was added to drinking water at 1 % w v⁻¹.

For pro-oxidant studies, male 12 week-old Zucker rats (Harlan, Blackthorn, Bicester, UK) were treated daily with HQ (50 mg kg⁻¹ i.p.), BSO (50 mg kg⁻¹ i.p.) or HQ+BSO (each at 50 mg kg⁻¹ i.p.) for 7 days. Control animals received sham injections of normal saline (2 mg kg⁻¹ i.p.) daily. HQ is a redox cycling compound that generates O₂^{•-}. Similarly, BSO is a

potent and selective irreversible inhibitor of reduced glutathione (GSH) synthetase and thereby results in the increased levels of ROS.

For fructose studies, male 7 week-old Zucker rats (Harlan, Blackthorn, Bicester, UK) were given untreated or fructose (10 %) (fructofin C) treated drinking water for 6 weeks. After 4 weeks of fructose treatment, pro-oxidant injections were started for 2 weeks as indicated above.

Rats were allowed food and water *ad libitum*. Obese and lean littermates were fasted overnight before vascular function was assessed and blood collected from the Zucker rats.

2.2.11 Measurement of plasma nitric oxide (Griess Assay)

Nitric oxide release was determined by measuring the accumulation of its stable degradation products, nitrate (NO_3^-) and nitrite (NO_2^-). Firstly, nitrate was reduced to nitrite, by incubating the samples for 15 min at 37°C in the presence of NADH or NADPH-dependent nitrate reductase (1 U ml^{-1}), NADPH (500 μM) and FAD (50 μM). When the nitrite reduction was complete, an additional step to degrade the excess NADPH was included to avoid its interference in the final reaction. This included the addition of lactate dehydrogenase (100 U ml^{-1}) and sodium pyruvate (100 mM) at 37°C for 5 min. The total nitrite was then determined spectrophotometrically using the Griess reaction – used with *N*-1-naphthylethylenediamine dihydrochloride under acidic (phosphoric acid) conditions to convert sulphanilamide to the purple-coloured azo compound that was measured at 550 nm in an Anthos Labtech type 12605 microplate reader.

2.2.12 Measurement of plasma total antioxidant status (TAOS)

The principle reaction of this microassay is the conversion of H_2O_2 to water which is coupled to the oxidation of 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid (ABTS) to ABTS^+ (Arnao *et al.*, 1996). Antioxidants present in the test samples will donate an electron to ABTS^+ , reducing it back to ABTS. Hence total antioxidant status of the test plasma is proportional to its ability to inhibit ABTS^+ generation. The reaction mixture consisted of ABTS (2 mM), horse radish peroxidase (30 mU ml^{-1}), H_2O_2 (0.1 mM), phosphate-buffered saline (10 mM, pH 7.4) and plasma. The reaction was initiated by the addition of H_2O_2 and conducted at 37°C. The increase in absorbance at 405 nm, reflecting the accumulation of ABTS^+ , was determined after 6 min in an Anthos Labtech type 12605 microplate reader. An ascorbate concentration curve was constructed and the data expressed as the ascorbate equivalent antioxidant concentration (AEAC). All determinations were made in triplicate.

2.2.13 Measurement of plasma insulin

Plasma insulin was determined using a commercial radioimmunoassay (RIA) kit (Linco Research Inc, Missouri, USA). Plasma, or known insulin standards, were added to rat ^{125}I -insulin and guinea pig anti rat insulin serum and incubated at 4°C for 24 hr. Competition between labelled tracer and unlabelled antigen for the antibody was stopped by the addition of an ice cold precipitating reagent containing goat anti-guinea pig IgG serum. The samples were vortexed and left to precipitate for 20 min at 4°C, before being centrifuged at 3000 rpm for 30 min. The precipitated pellets were counted in a gamma counter (NE160, Nuclear Enterprises Ltd, Edinburgh, UK), and insulin concentrations in samples calculated from the standard curve, where (%) standard/total counts was plotted against its concentration. All determinations were made in duplicates. The limit of sensitivity of the assay is 0.1 ng ml^{-1} , and the limit of linearity is 10 ng ml^{-1} .

2.2.14 Measurement of plasma glucose

Plasma glucose was measured using a commercial photometric Trinder assay kit (Sigma Chemical Co. Poole, UK.). The volumes and plasma dilution recommendations were modified from the manufacturer’s instructions to determine glucose concentrations in a microtitre plate. The principle reactions in the assay are, firstly, the oxidation of glucose to gluconic acid and H₂O₂ by glucose oxidase. The H₂O₂ formed then reacts with 4-aminoantipyrine and p-hydroxybenzene sulfonate in the presence of peroxidase to form a quinoneimine dye, with an absorbance maximum at 505 nm. The colour intensity is directly proportional to the glucose concentration in the sample. The assay procedure for the microassay was to add the standards (1.25-40 mM) or plasma samples to a 96 well plate and quickly add the light sensitive trinder reagent. The plate was covered in foil, shaken and incubated at room temperature for 18 min. The plasma glucose concentration was determined by absorbance at 505 nm in an Anthos Labtech type 12605 microplate reader. Since turbid as well as haemolyzed plasma may give falsely high values, blank absorbance was determined for each plasma sample and these values were subtracted from the actual sample values. All determinations were made in duplicates.

Standard (mM)	0	1.25	2.5	5	10	20	40
% CV	2.52	5.23	3.47	4.64	4.41	5.89	5.63

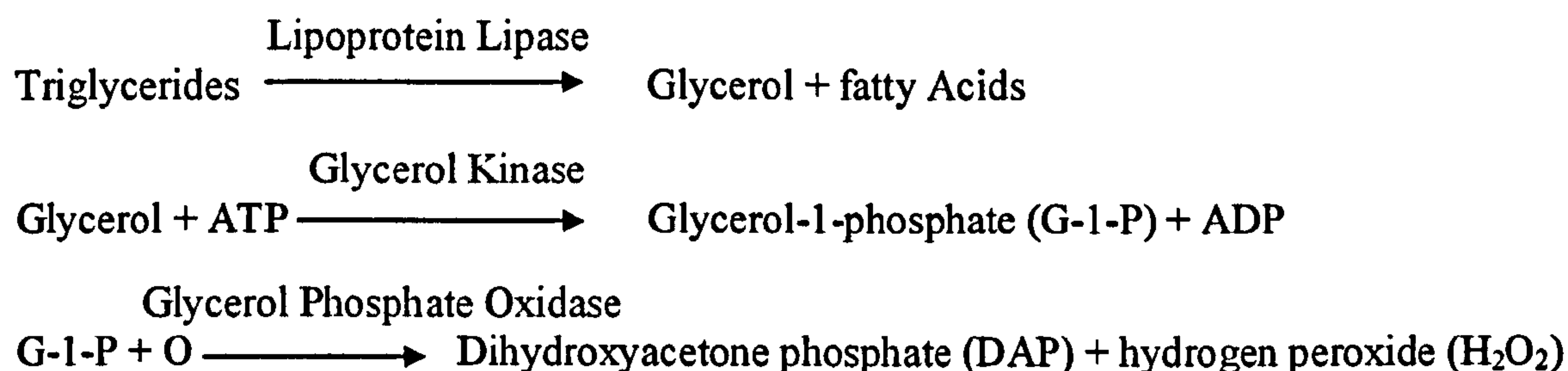
Table 2.1 Intra sample variation in the glucose standards (0-40 mM) in saline

Plasma (mM)	0	5	10
% CV	3.98	4.53	4.31

Table 2.2 Inter assay variation in glucose spiked plasma samples (0-10 mM)

2.2.15 Measurement of plasma triglycerides

Triglycerides were measured using a commercial photometric triglyceride assay kit – Triglycerides GPO-Trinder (Sigma Chemical Co. Poole, UK.) which was carried out according to the kit instructions. Triglycerides, esters of fatty acids and glycerol do not circulate freely in plasma but are bound to proteins and transported as macromolecular complexes called lipoproteins. Therefore there are a number of enzymatic reactions in the assay.



Finally, a quineimine dye is produced by the peroxidase catalysed coupling of 4-aminoantipyrine (4-AAP) and sodium N-ethyl-N-(3-sulfopropyl) m-anisidine (ESPA) with H_2O_2 which shows an absorbance maximum at 540 nm. The increase in absorbance at 540 nm is directly proportional to triglyceride concentration of the sample. The triglyceride (GPO-Trinder) reagent solution was warmed to assay temperature and added to a triglyceride standard or plasma. The sample was mixed and incubated at 37°C for 5 min. Following incubation the absorbance was measured at 540 nm in a spectrophotometer (Shimadzu UV-160, Howe, Oxon, UK). All determinations were made in duplicates.

2.3 DATA ANALYSIS

Data are presented as means \pm s.e.mean of n independent experiments, except in the glucose assay method validation where s.d was used to calculate the coefficient of variation (% CV) for intra and inter assay variation (tables 2.1 & 2.2). Statistical analysis between paired means were assessed using Student's two-tailed, paired t -test and a P value of less than

0.05 was considered statistically significant. Multiple comparisons of unpaired mean dose response curves from functional data shown by graphs were conducted by two way ANOVA followed by a Dunnett's test. All other multiple comparisons of unpaired means from functional data were conducted by one way ANOVA followed by a Dunnett's test. Data was analysed using Prism (GraphPad software Inc., U.S.A.). Significance was accepted at the 5 % level. ACh-induced changes in perfusion pressure are expressed as a percentage of the total vascular response (% SNP) in order to control for changes in the preparations. The figures are presented as means \pm s.e.mean. Error bars not represented are smaller than the symbol size used.

Chapter 3

*Development of methods for the
investigation of endothelial function*

3.1 INTRODUCTION

Since the importance of endothelial function in the pathogenesis of diabetes remains unclear, a key goal of this work was to develop appropriate methods to assess endothelial function in rat models of diabetes. Many studies measuring forearm blood flow in type 2 diabetic patients have shown impaired endothelium-dependent responses. However, studies with uncomplicated type 2 diabetic patients showed no difference in basal or agonist stimulated NO (Avogaro *et al.*, 1997). This picture is further complicated by studies in animals with early diabetes that show an increased formation of endothelial-derived NO (Corbett *et al.*, 1992; Tilton *et al.*, 1993). Conflicting data in this area may be due firstly to the size of the vessel where a given measurement is taken. For example, NO is a key vasodilator in large arteries, but has a diminishing role as vessel calibre decreases (Urakami-Harasawa *et al.*, 1997). Secondly, some measurements are made in more insulin sensitive areas than others – an aortic ring is less sensitive to insulin than a skeletal muscle bed. Thirdly, differences in *in vitro* and *in vivo* data will occur as blood borne factors and autonomic control will affect the measurement of endothelial function. The importance of using a variety of systems to measure endothelial function is therefore paramount to investigate its role in diabetes.

Development of a rat model to assess blood flow and pressure simultaneously in vivo. Previous assessment of endothelial function in the rabbit ear artery using photoplethysmography (Weinberg *et al.*, 2001) has shown NO-dependent changes in blood flow induced by ACh. Similar studies in humans showed NO-dependent changes in blood flow were impaired in type 2 diabetes patients (Chowienczyk *et al.*, 1999). Development of a rat model would allow correlation of agonist-induced changes in blood flow in a small artery with overall changes in blood pressure. The blood flow measurements can be obtained using laser Doppler fluxmetry focused on the auricular ear artery. Principally, this

works by measuring frequency changes in a light beam that has been scattered in response to moving red blood cells. This method subsequently provides a measurement of red blood cell flow in a given volume, as the higher the frequency, the greater the amount of red blood cells. Hence a vasodilation of the ear artery results in less vascular resistance, and consequently an increased Doppler frequency.

The model was additionally developed for its potential use as a non-invasive measurement of endothelial (dys)function so that animals could be used in long term studies. This would limit animal usage and allow better numerical data as intra variation analysis could be done. A further advantage of this approach is that the use of anaesthetic would be limited – it is well documented that anaesthesia can affect endothelial function (Castillo *et al.*, 1999). Since any potential non-invasive method of endothelial function measurement would require drug administration via the tail vein, a comparison was made of tail verses jugular vein drug administration in respect of changes in arterial blood flow.

Rat tail indirect blood pressure system provides a non-invasive method for blood pressure measurement and is therefore suitable for long term studies. Furthermore, this method is considered appropriate to use in parallel with non-invasive measurements of ear arterial blood flow, as blood pressure and blood flow could be measured simultaneously. The new system required testing in our laboratory, as rat body temperature and stress can affect the reliability of data. It was my aim to carry out the method at room temperature, because increasing the body temperature may alter endothelial function due to vasodilation. Previous studies have used rats that were warmed to 37°C, a practice that increased the blood flow to the tail and allowed the detection of a pulse. Additionally, rats need to become accustomed to being held in restrainers to reduce stress-induced constriction of their tails (Bunag & Butterfield, 1982).

The perfused rat hindquarters in situ is an ideal method for measurement of endothelial function in studies of diabetes, since the hindquarters have a large resistance vascular area that is insulin sensitive. This *in situ* preparation thus allows the direct measurement of endothelium-dependent mechanisms from changes in pressure and flow without the complications of autonomic nervous control and blood. For example, cardiovascular autonomic baroreceptor reflexes increase sympathetic activity in response to acute decreases in blood pressure in order to maintain homeostasis. Equally problematical is the sensitivity of blood borne factors, such as catecholamines, the activity of which can be increased by as much as 200 fold in blood-perfused systems. In addition, haemoglobin in the circulating blood may provide a constrictor vascular tone by binding some of the NO that is continually released from the endothelium (Martin *et al.*, 1986).

The haemodynamic relationship between blood flow, pressure and vessel tone (diameter) in both the perfused hindquarters *in situ* and ear blood flow experiments is described by Poiseuille's equation (Nichols & O'Rourke, 1998): $Q = KD^4 (P_1 - P_2) / L$; where Q= volume flow of blood or perfusate per unit time, K is a constant, D the internal diameter of the vessel (which is dependent on vasodilation), and $(P_1 - P_2)$ is the pressure drop (or difference) along L (the length of the vessel). This was derived from the equation Pressure = Flow x Resistance (vessel diameter). There is some debate as to the type of perfusion model used. For example, flow can be kept constant and pressure changes recorded, or pressure can be kept constant and flow is varied according to the dilation of the preparation (Johnsson *et al.*, 1991). Despite the reduction in oedema formation in the constant pressure models, the flow is a more preferred method as the delivery of drugs is more problematical in the constant pressure model, because the concentration at a particular flow cannot be predicted. Consequently, this more sensitive model can be harder to interpret.

3.2 METHODS

3.2.1 Perfusion of the rat hindquarters *in situ*

The surgical procedures used to set up this method are included in section 2.1.1 of the general methods chapter.

In initial validation studies, the effluent outflow rate and basal perfusion pressure (PP) were determined at 30 min intervals over a perfusion period of 150 min in order to assess variation with time. In addition, vasoconstrictor reactivity was assessed over this time course by construction of a dose response curve for vasoconstriction to bolus doses of phenylephrine (Phe) (1-100 nmol).

In basal endothelial function studies, the hindquarters received cumulative concentrations of Phe (0.3-1000 nmol min⁻¹) by infusion, with or without L-NAME (2.1 µmol) in the PSS, for 20 min before and during the Phe response curve.

In agonist stimulated endothelial function studies, Phe eliciting approximately 85% of the maximal response (Wistar rat: 300 nmol min⁻¹) was infused at t=20 min to provide sub-maximal pre-constriction. After a stabilisation of induced vascular tone, bolus doses of ACh (0.001-30 nmol) or SNP (0.001-100 nmol) were injected to construct vasodilator dose response curves.

In separate experiments, the effect of L-NAME on an ACh dose response curve was assessed at t=20 min in hindquarters preparations pre-constricted with Phe (300 nmol), in the presence or absence of L-NAME (2.1 µmol) in the PSS. After a stabilisation of induced vascular tone, bolus ACh was administered as above. In these experiments, SNP (100

nmol) was injected to construct vasodilator dose response curves with respect to SNP (as an internal control and an estimation of the total vasodilation ability of the preparation).

3.2.2 Haemodynamic measurements *in vivo*

Blood pressure. Male Wistar rats were anaesthetised with thiopentone sodium (Intraval[®], 120 mg kg⁻¹, i.p.). The trachea was cannulated to facilitate respiration and the rectal temperature was maintained at 37°C as described above. The right carotid artery was cannulated and connected to a pressure transducer (Sensonor 840, Norway) for the measurement of mean arterial blood pressure (MAP) and heart rate (HR) which were recorded for the duration of the experiment and displayed on a MacLab 16s recording system (AD Instruments, London, UK). The femoral vein was cannulated for the bolus administration of drugs, and the jugular vein for the infusion of drugs.

Blood flow. In blood flow experiments, the surgical procedure for blood pressure measurements was completed before shaving the ear of the rat and placing a laser Doppler flow probe (Type P4; tip diameter 0.85 mm; Moor Instruments, Axminster, Devon, UK) on the ear auricular artery, or a branch of the artery (Fig. 3.1). Blood flow was also displayed on the MacLab recording system. Following surgery, a 30 min period of stabilisation was allowed before recording baseline haemodynamic parameters.

ACh (0.001-10 nmol kg⁻¹ i.v.) and SNP (0.01-30 nmol kg⁻¹ i.v.) were administered to assess vasodilation and changes in blood flow mediated by endogenous or exogenous NO respectively. Separate experiments were performed to establish the role of NO in blood flow in the auricular artery of the ear. Agonist-stimulated NO responses were assessed by bolus ACh (1&10 nmol kg⁻¹ i.v.) responses. SNP (10 nmol kg⁻¹ i.v.) was administered for both the assessment of endothelial-independent NO vasodilation, and as an internal control.

L-NAME was administered initially as a bolus ($50 \text{ mg kg}^{-1} \text{ i.v.}$), followed by a 10 min infusion ($100 \text{ mg kg}^{-1} \text{ hr}^{-1} \text{ i.v.}$ Moncada *et al.*, 1991). Cardiovascular responses to bolus doses of ACh then SNP were then reassessed.

ACh-induced vasodilation and changes in blood flow are expressed as a percentage of the total vascular response (% SNP) to control for probe movement during the experimental procedure. The vasodepressor activity and changes in blood flow are plotted as the area under the response curve with time as (mm Hg min^{-1}) and (V s^{-1}) respectively.

Vasoconstrictor reactivity was assessed *in vivo* by construction of a dose response curve to bolus doses of Phe ($0.1\text{-}100 \text{ nmol kg}^{-1} \text{ i.v.}$). In agonist stimulated endothelial function studies, bolus doses of ACh ($0.01\text{-}10 \text{ nmol kg}^{-1} \text{ i.v.}$) or SNP ($1\text{-}100 \text{ nmol kg}^{-1} \text{ i.v.}$) were injected to construct vasodilator dose response curves.

3.2.3 Rat tail cuff blood pressure measurement

Rats were placed in a restrainer, and a cuff was placed at the base of the tail. The rats were then allowed to acclimatize to their surroundings for 20-30 min prior to cuff inflation. Using a manual inflation bulb, the cuff was gently inflated to 250-300 mm Hg to occlude blood flow. A bleed valve was then opened to reduce the pressure over a 15 second period. The inflation pressure and the tail pulse pressure were recorded using a MacLab system (Hastings, Sussex, U.K.). The recorded blood pressure measurement is the mean of 5 individual readings taken 5 min apart. Analysis of the traces show that as the inflation pressure decreases a point will be reached where the pulse pressure will become apparent on the trace, this is read as the systolic pressure. As the inflation pressure decreases further, the amplitude of the pulse pressure will rise and then plateau, this plateau is read as MAP (Fig. 3.2).

3.3 RESULTS

3.3.1 Development of an *in situ* hindquarters perfusion model.

Initial validation studies. The effluent outflow rate decreased slightly over the initial 90 min, with a sharp decrease occurring after this time (Fig. 3.3A). In contrast, basal perfusion pressure exhibited only a slight rise over 150 min (approximately 10 mm Hg) (Fig. 3.3C). Hindquarters basal perfusion pressure after $t=20$ min was 22.8 ± 1.2 mm Hg ($n=35$). Vasoconstrictor responsiveness to Phe (10 nmol) moderately increased between $t=30$ and $t=60$ min and then remained reproducible until $t=120$ min, after which time there was a further significant increase in sensitivity (at $t=150$ min) (Fig. 3.3B).

L-NAME enhances Phe-induced vasoconstriction. Basal perfusion pressure of the control preparations (28.0 ± 1.7 mm Hg, $n=7$) was comparable to that of L-NAME (27.7 ± 2.5 mm Hg, $n=5$) treated preparations ($P>0.05$). However, Phe (0.3 - 1000 nmol min^{-1}) elicited graded increases in perfusion pressure that were shifted leftward in the presence of L-NAME (2.1 μmol) (Fig. 3.4A).

ACh and SNP-induced vasodilation. Basal perfusion pressures in ACh (28.0 ± 1.4 mm Hg, $n=4$) and SNP preparations (28.3 ± 4.1 mm Hg, $n=4$) were elevated to sustained perfusion pressures by Phe (ED_{85} , 300 nmol min^{-1}) (ACh preparations: 182.2 ± 18.2 mm Hg, $n=4$; SNP preparations: 86.3 ± 19.0 mm Hg, $n=4$). Vasodilator responses to ACh (0.0001 - 30 nmol) (Fig. 3.4B) and SNP (0.001 - 100 nmol) (Fig. 3.4C) elicited rapidly reversible, dose-dependent decreases in perfusion pressure.

L-NAME inhibits ACh-induced vasodilation. Basal perfusion pressure in control preparations (28.3 ± 2.9 mm Hg, $n=6$) was comparable with that of L-NAME treated preparations (32.8 ± 2.5 mm Hg, $n=6$) ($P>0.05$). However, Phe elevated perfusion pressure

was greater in L-NAME (255.7 ± 5.7 mm Hg, $n=6$; $P<0.05$) relative to control preparations (157.1 ± 27.9 mm Hg, $n=6$). Furthermore, the ACh (0.0001 - 100 nmol) -induced vasorelaxation was impaired by the administration of L-NAME (2.1 μ mol) ($P<0.05$) (Fig. 3.5). In contrast, the SNP (100 nmol) bolus dose elicited a greater vasorelaxation in the L-NAME (144.5 ± 14.8 %, $n=6$; $P<0.05$) treated preparations compared to the control (87.4 ± 13.9 %, $n=6$) preparations.

3.3.2 Development of a non-invasive technique for ear artery blood flow measurement

ACh and SNP -induced dose-dependent changes in MAP and blood flow. The administration of endothelium-dependent and -independent vasodilators provoked a decrease in MAP that was seen to be followed by a simultaneous increase in pulsatile blood flow in the ear artery (Fig. 3.1). Bolus administration of ACh dose-dependently elicited both a decrease in MAP and a simultaneous increase in blood flow in the auricular artery of the rat ear (Fig. 3.6) from baseline values of 126.28 ± 5.76 mm Hg ($n=12$) and 4.42 ± 0.16 V ($n=12$) respectively. Similarly, SNP (0.01 - 30 nmol kg^{-1}) induced both an increase in blood flow and decrease in MAP in a dose-dependent manner (Fig. 3.7). However, administration of ACh or SNP did not alter the basal heart rate (HR) of 380.15 ± 11.08 beats min^{-1} (BPM) ($n=12$).

The correlation coefficient (r) for ACh (0.001 - 10 nmol kg^{-1}) dose responses showed a 71 % (correlation $P<0.05$) similarity of blood flow to the MAP ($n=6$; data points included=36). However, a significantly greater relationship between flow and MAP of 88 % (correlation $P<0.05$) was observed in the dose responses to SNP (0.01 - 30 nmol kg^{-1}) ($n=6$; data points included=22) (Fig. 3.8A; Fig. 3.8B) when the slopes were compared directly. ACh and SNP induced similar changes in MAP ($P>0.05$); changes in blood flow, however, were more sensitive to ACh than SNP ($P<0.05$, Two way ANOVA).

L-NAME inhibited ACh-induced changes in MAP but not blood flow. ACh-induced (1 & 10 nmol kg⁻¹) increases in ear artery blood flow were unaffected by L-NAME (Vehicle; $n=5$, L-NAME; $n=6$) (Fig. 3.9). In contrast, the decrease in MAP to ACh was significantly impaired by L-NAME ($n=5-6$) (Fig. 3.10). Furthermore, SNP-induced changes in basal blood flow (0.89 ± 0.26 V, $n=6$), MAP (33.96 ± 3.40 mm Hg, $n=6$), and HR (21.46 ± 13.49 BPM, $n=5$) (10 nmol kg⁻¹) were unaffected by L-NAME treatment (flow; 1.46 ± 0.21 V, $n=6$, MAP; 31.97 ± 8.9 mm Hg, $n=6$, HR; 2.76 ± 4.56 BPM, $n=5$). Infusion of L-NAME alone decreased ear blood flow, but this was not statistically significant (absolute fall: 4.4 ± 0.5 V to 2.4 ± 0.8 V, ($n=6$); area under the response curve: -343 ± 149 V s⁻¹ to 1285 ± 822 V s⁻¹). Additionally, the increases in MAP (increase of 9.56 ± 5.0 mm Hg, $n=6$) and the decrease in HR (decrease of 32.95 ± 12.17 BPM, $n=6$) by L-NAME were not statistically significant.

Route of administration altered ACh-induced changes in MAP but not blood flow. ACh-induced (10 nmol kg⁻¹) increases in ear artery blood flow were unaffected by route of administration (femoral; $n=6$, tail; $n=6$) (Fig. 3.11A). In contrast, the decrease in MAP to ACh was significantly impaired when administered via the tail vein ($n=6$) (Fig. 3.11B). In addition, SNP-induced (10 nmol kg⁻¹) changes in basal blood flow ($n=6$), MAP ($n=6$), were unaffected by route of administration.

3.3.3 Non-invasive measurement of tail artery blood pressure.

Basal measurement of MAP. The basal arterial blood pressure measurement using the non-invasive tail cuff was 117.85 ± 5.3 mm Hg ($n=5$). Similarly, direct invasive MAP measurement was essentially the same 114.23 ± 2.5 mm Hg ($n=5$) in anaesthetised rats (Fig. 3.12).

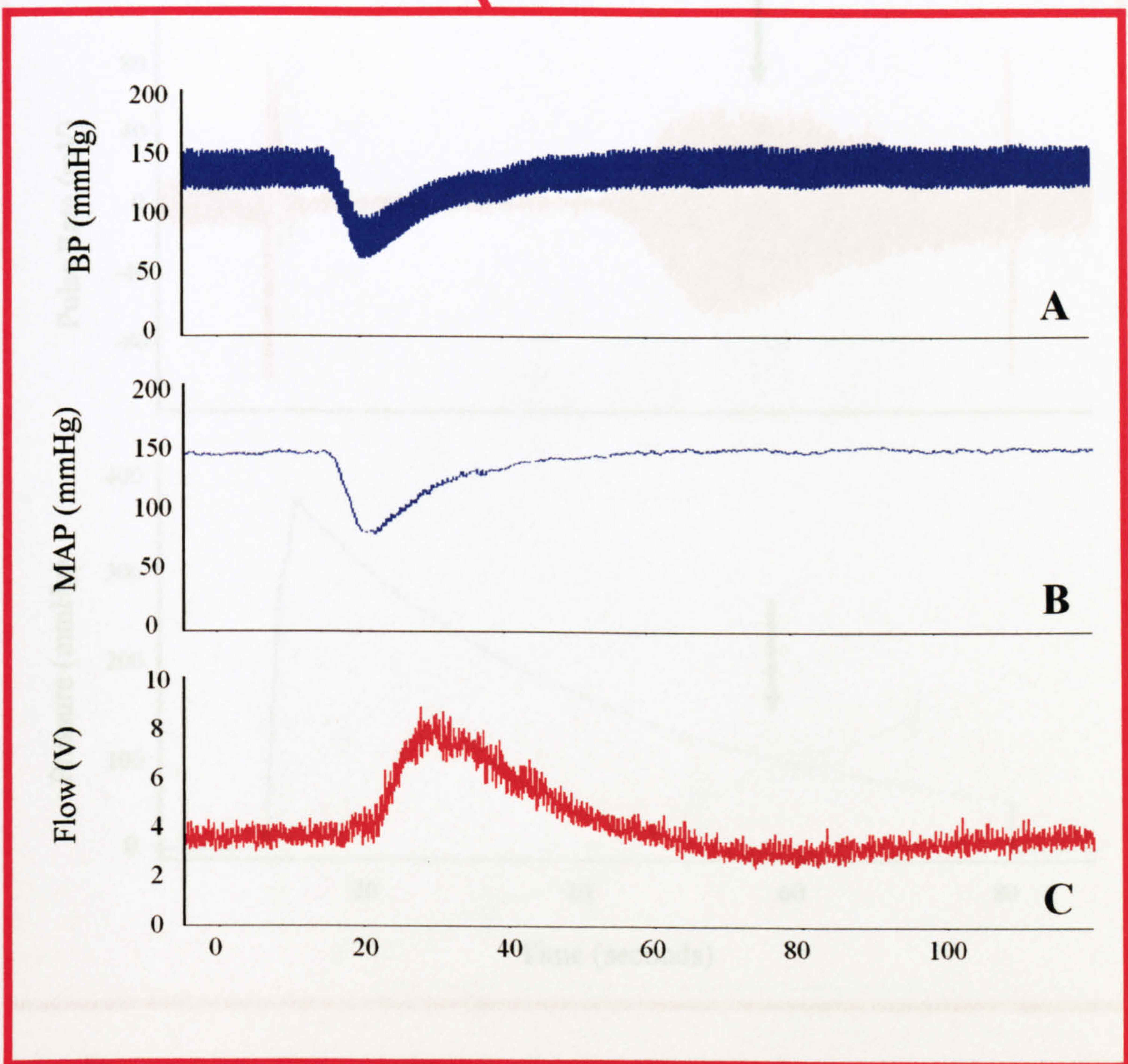
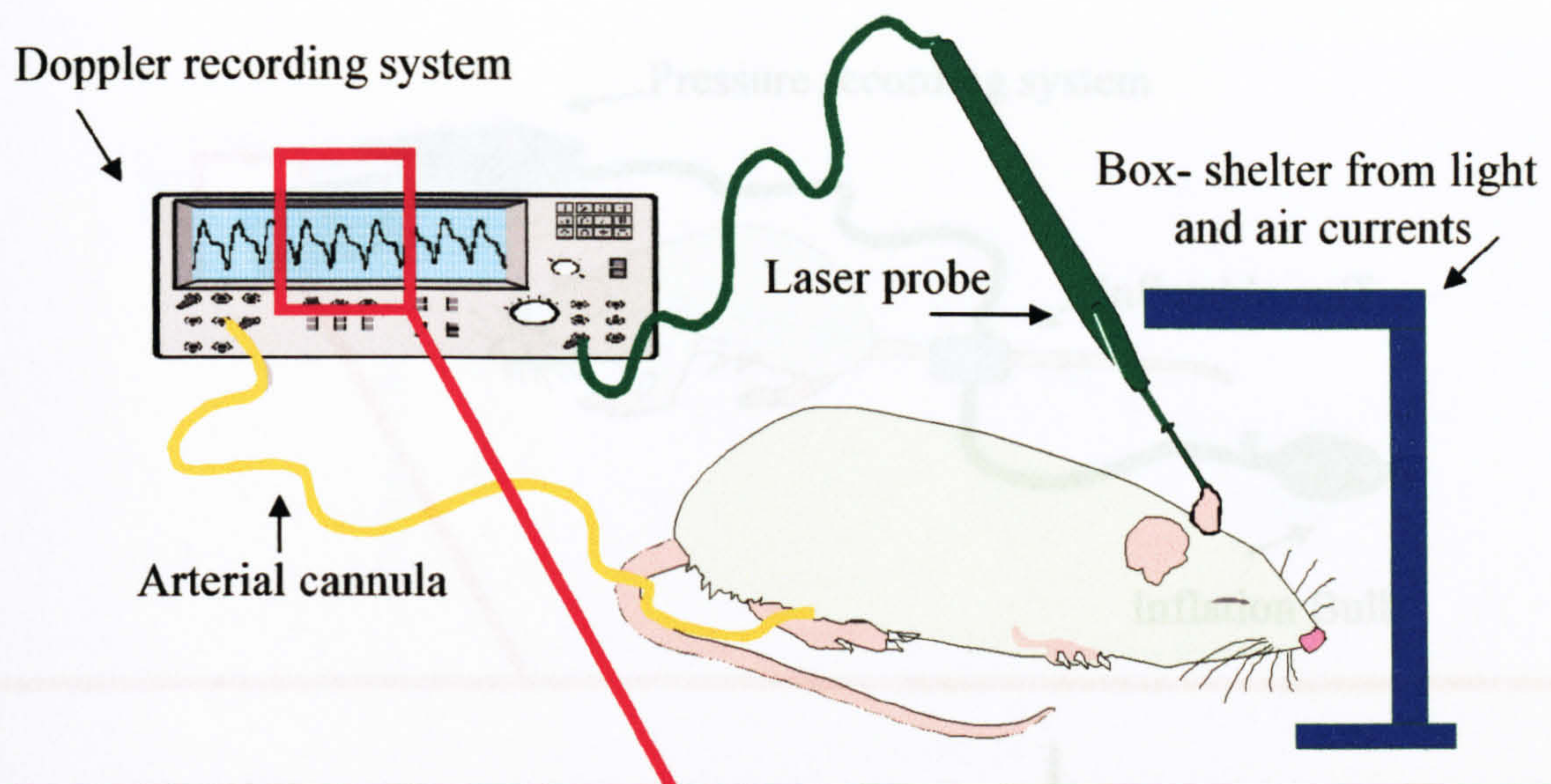


Figure 3.1 Experimental setup and typical traces for the measurement of ear blood flow using laser Doppler fluximetry. Mean arterial blood pressure was measured using traditional pressure transducers. The traces show the A. blood pressure (■) B. MAP C. blood flow (■) following a bolus dose of acetylcholine. The computer recorded at 40 samples per second.

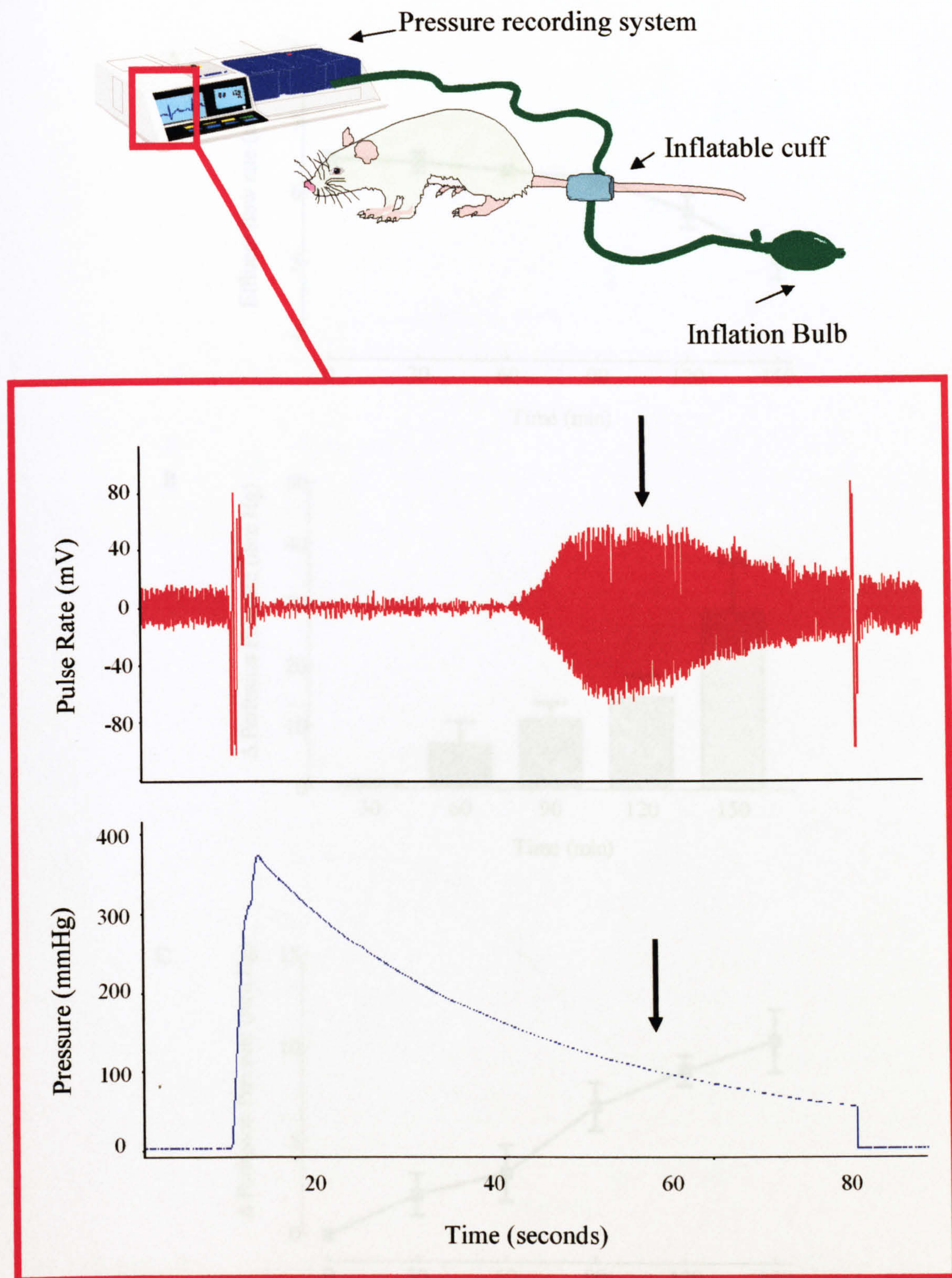


Figure 3.2 Typical trace using the tail cuff for indirect measurement of mean arterial blood pressure in the conscious rat. The traces show the occlusion and gradual release of the cuff pressure (■) and simultaneous threshold then regular pulse rate (■). The arrow indicates where the mean arterial blood pressure value was measured. The computer recorded at 200 samples per second.

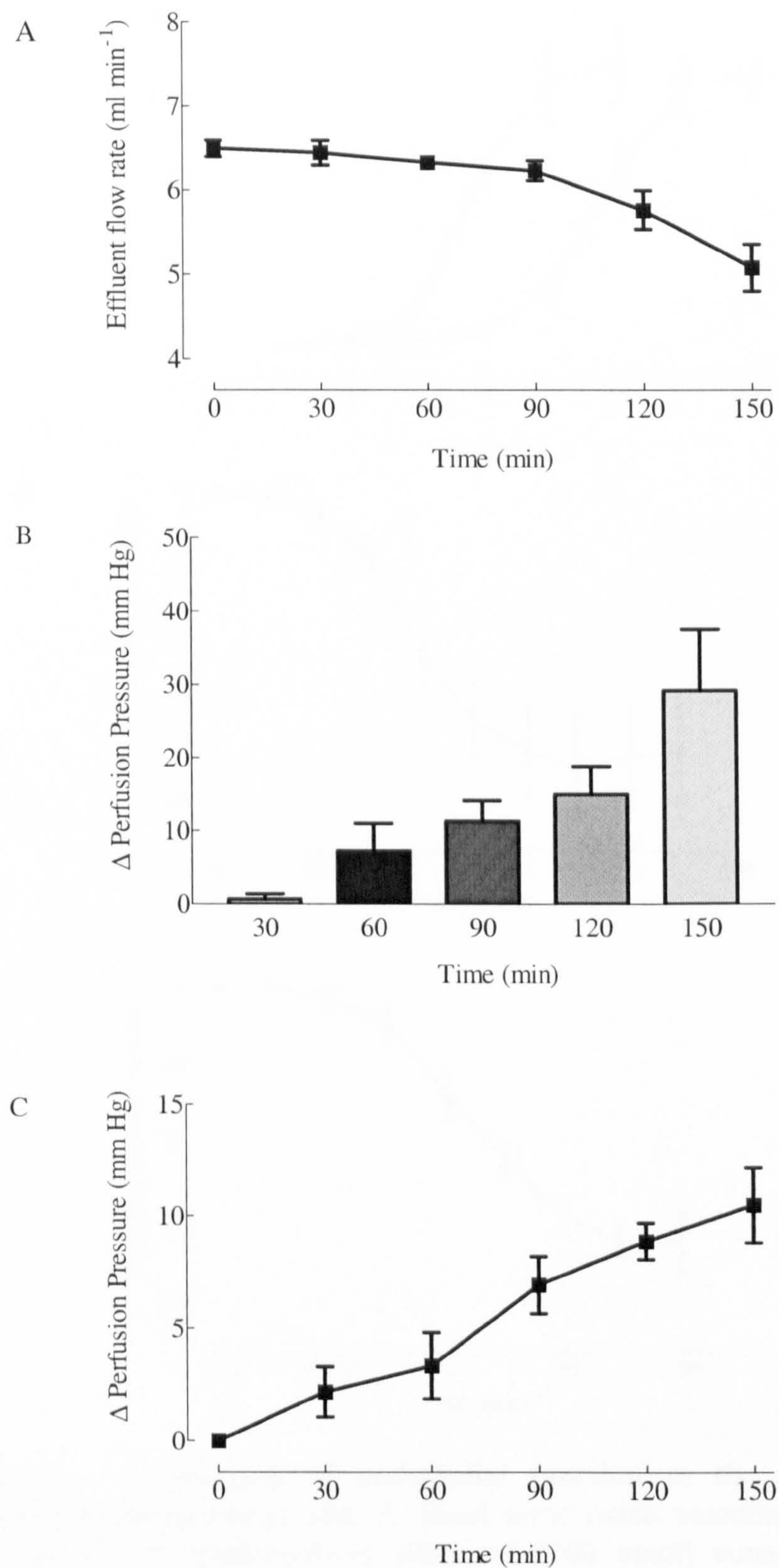


Figure 3.3 Initial validation studies of the Wistar rat perfused hindquarters *in situ* included the effect of perfusion time on: A. Effluent flow rate. B. Reactivity of preparation to Phenylephrine (10 nmol) -spiked responses. C. Basal perfusion pressure. Data are expressed as mean \pm s.e.mean of 3 determinations.

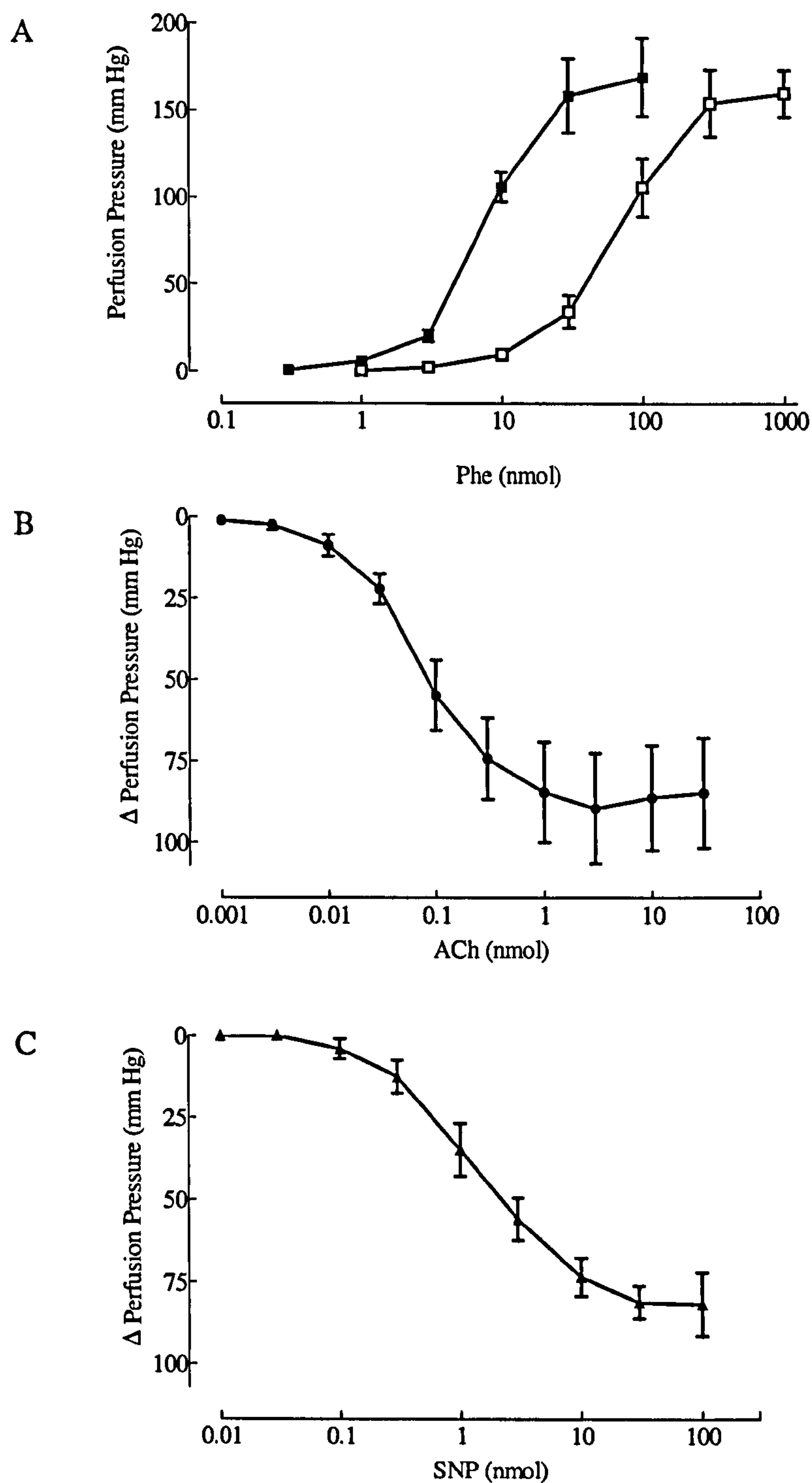


Figure 3.4 Assessment of endothelial function in the Wistar rat perfused hindquarters *in situ*. A. Basal nitric oxide vasodilator function was assessed by phenylephrine (Phe, 0.1-1000 nmol) cumulative dose responses in control (□) and L-NAME (2.1 μ mol equivalent to 300 μ M) (■) treated preparations. Established vasoconstrictor tone by phenylephrine (300 nmol) was reversed by the endothelium -dependent and -independent agonists B. Acetylcholine (ACh, 0.001-100 nmol) (●) C. Sodium nitroprusside (SNP, 0.01-100 nmol) (▲). Data are expressed as mean \pm s.e.mean of 4-8 determinations.

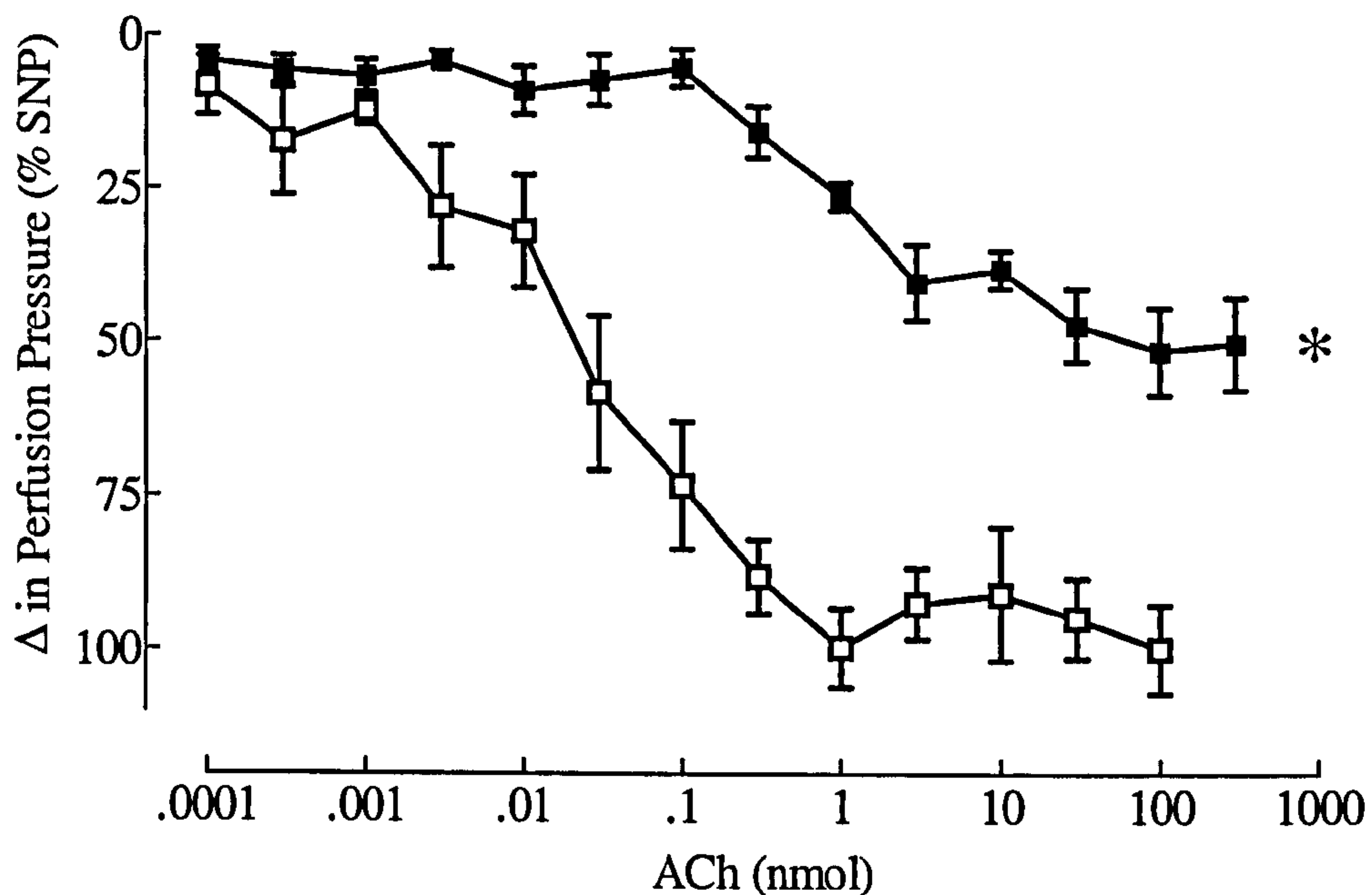


Figure 3.5 Vasodilation to acetylcholine (ACh, 0.0001-100 nmol) in phenylephrine (300 nmol) pre-constricted Wistar rat perfused hindquarter *in situ*. Preparations were control (□) and L-NAME (2.1 μmols) (■) treated. ACh was standardised with respect to vasodilation to sodium nitroprusside (SNP, 100 nmol) Data are expressed as mean±s.e.mean of 4-6 determinations. Statistical significance (*) using two way ANOVA was considered established at $P<0.05$

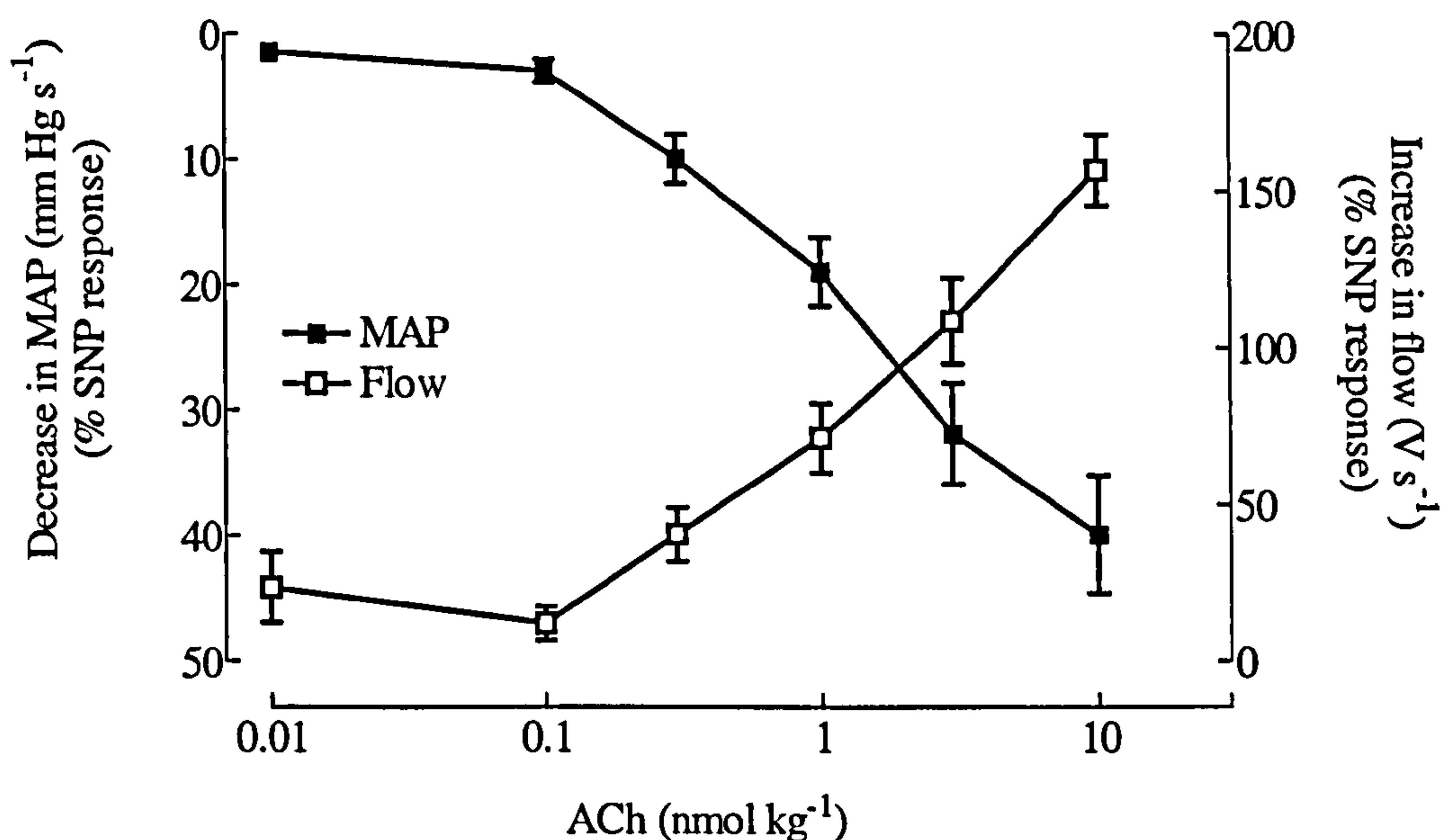


Figure 3.6 Acetylcholine (ACh, 0.001-10 nmol kg⁻¹ i.v.) -induced endothelium-dependent changes in mean arterial blood pressure (MAP) (■) or arterial blood flow (□) of the ear in the Wistar rat. Results are standardised with respect to vasodilation to sodium nitroprusside (SNP, 10 nmol kg⁻¹ i.v.). Data are expressed as mean±s.e.mean of 6 determinations.

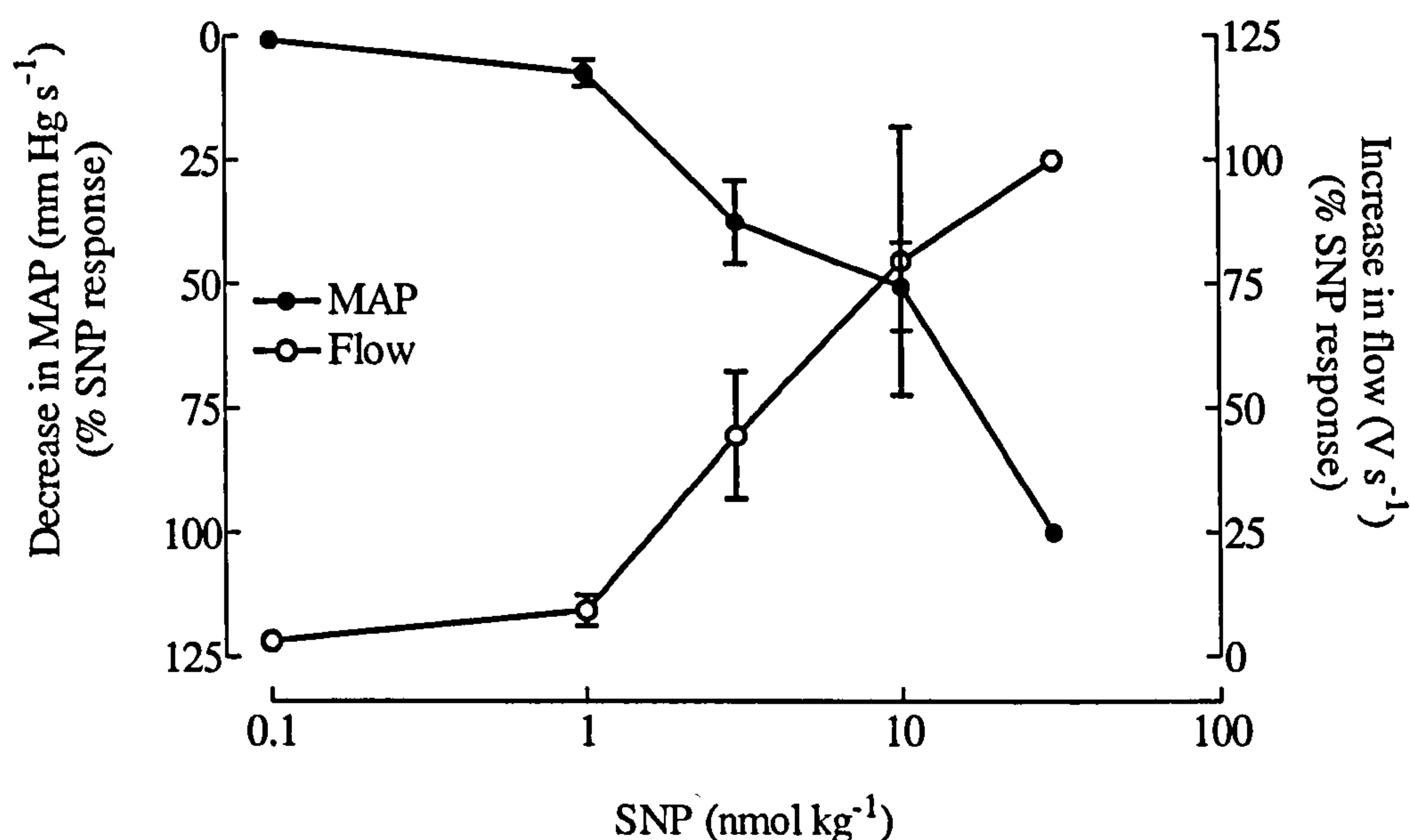


Figure 3.7 Sodium nitroprusside (SNP, 0.01-30 nmol kg⁻¹ i.v.) -induced endothelium-independent changes in mean arterial blood pressure (MAP) (●) or arterial blood flow (○) of the ear in the Wistar rat. Results are standardised with respect to vasodilation to sodium nitroprusside (SNP, 10 nmol kg⁻¹ i.v.). Data are expressed as mean±s.e.mean of 6 determinations.

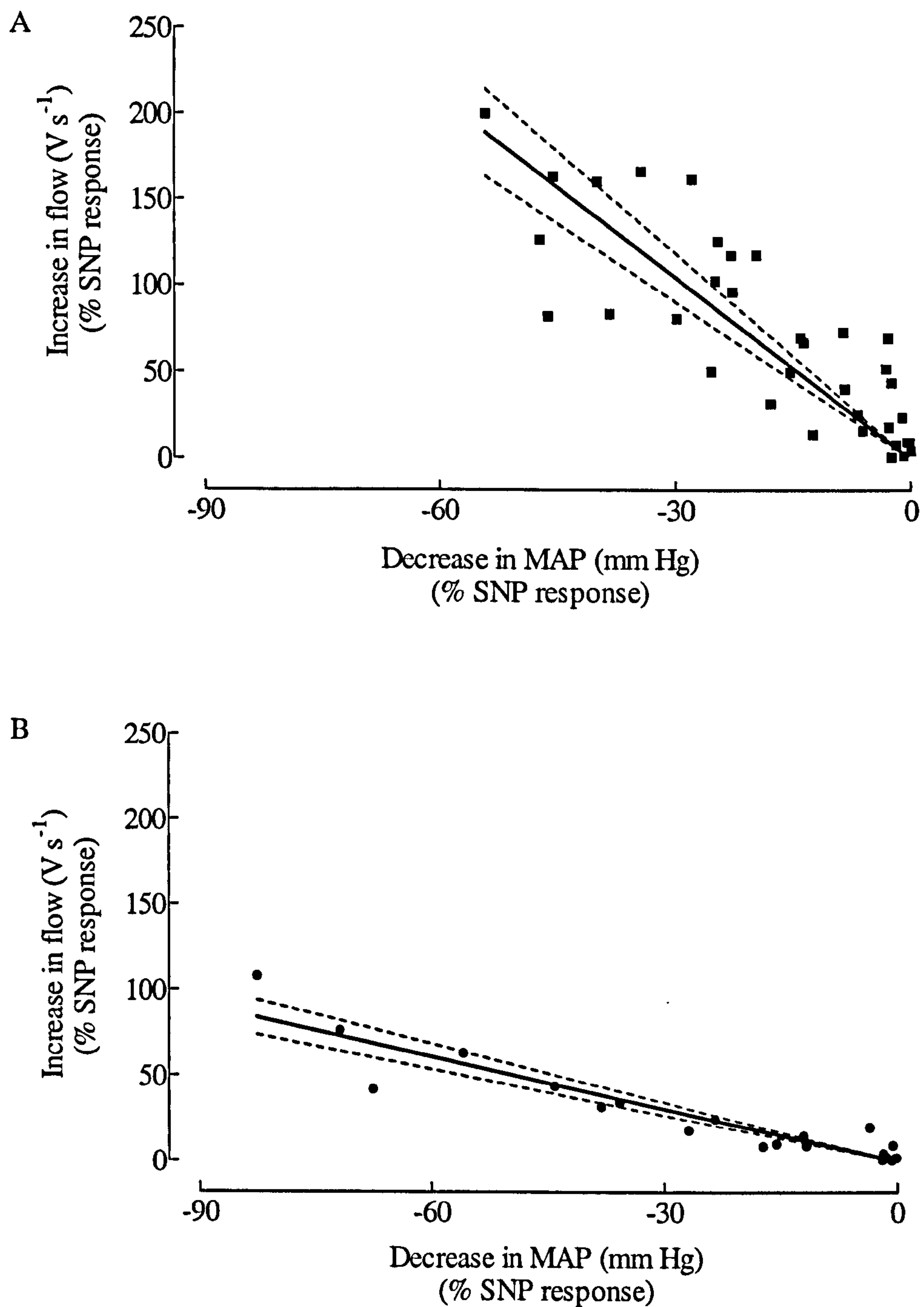


Figure 3.8 Correlation of (A). Acetylcholine (ACh, 0.001-10 nmol kg⁻¹ i.v.) or (B). Sodium nitroprusside (SNP, 0.01-30 nmol kg⁻¹ i.v.) -induced decrease in mean arterial blood pressure (MAP) and increase in ear arterial blood flow in the Wistar rat *in vivo*. Results are standardised with respect to vasodilation to SNP (10 nmol kg⁻¹ i.v.). Data are expressed as mean \pm s.e.mean of 6 determinations.

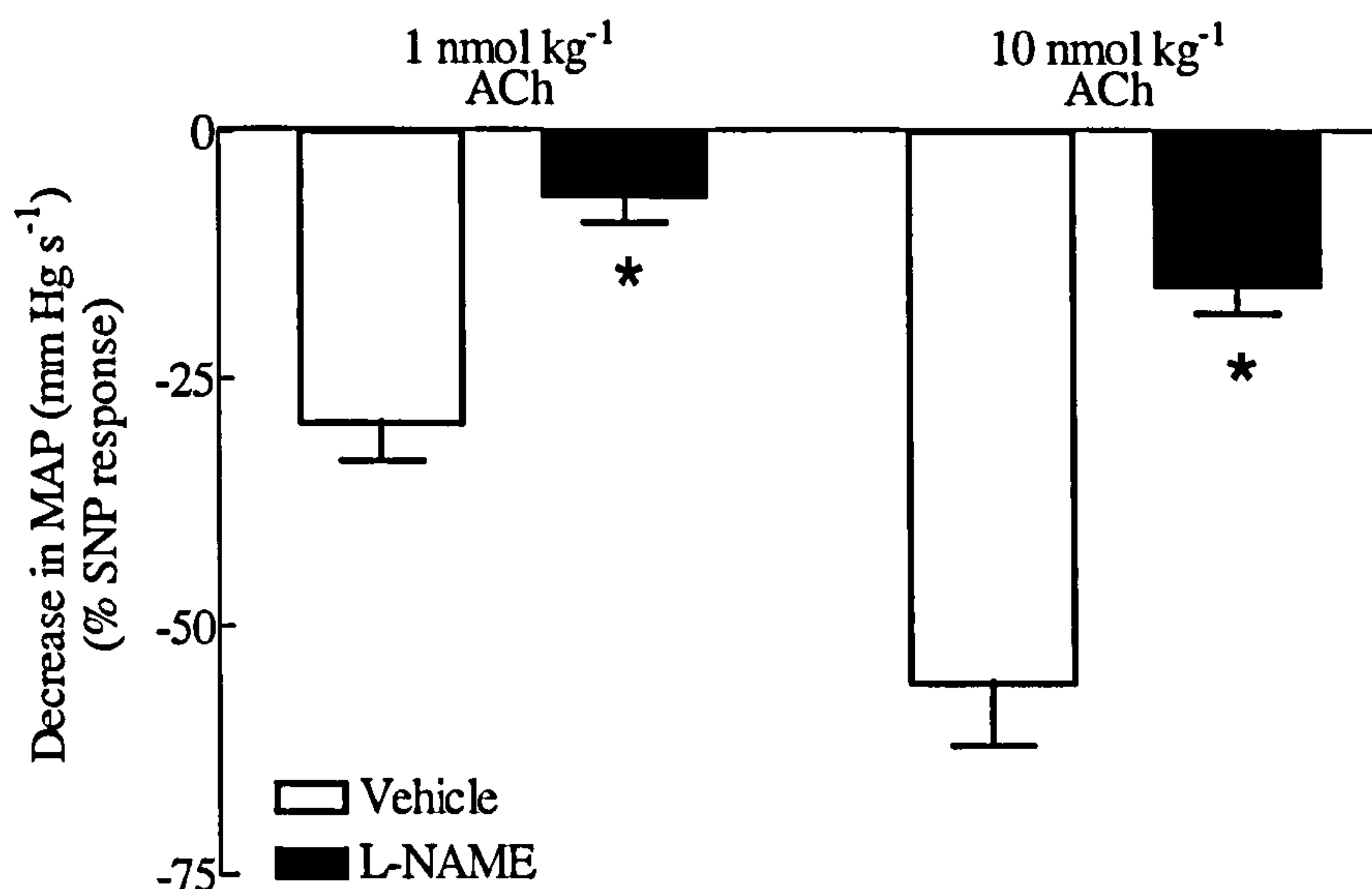


Figure 3.9 Acetylcholine (ACh, 1&10 nmol kg⁻¹ i.v.) -induced endothelium-dependent changes in mean arterial blood pressure (MAP) in vehicle (□) and L-NAME (100 mg kg⁻¹) (■) treated Wistar rats *in vivo*. Results are standardised with respect to vasodilation to sodium nitroprusside (SNP, 10 nmol kg⁻¹ i.v.). Data are expressed as mean±s.e.mean of 5-6 determinations. **P*<0.05 vs. saline vehicle.

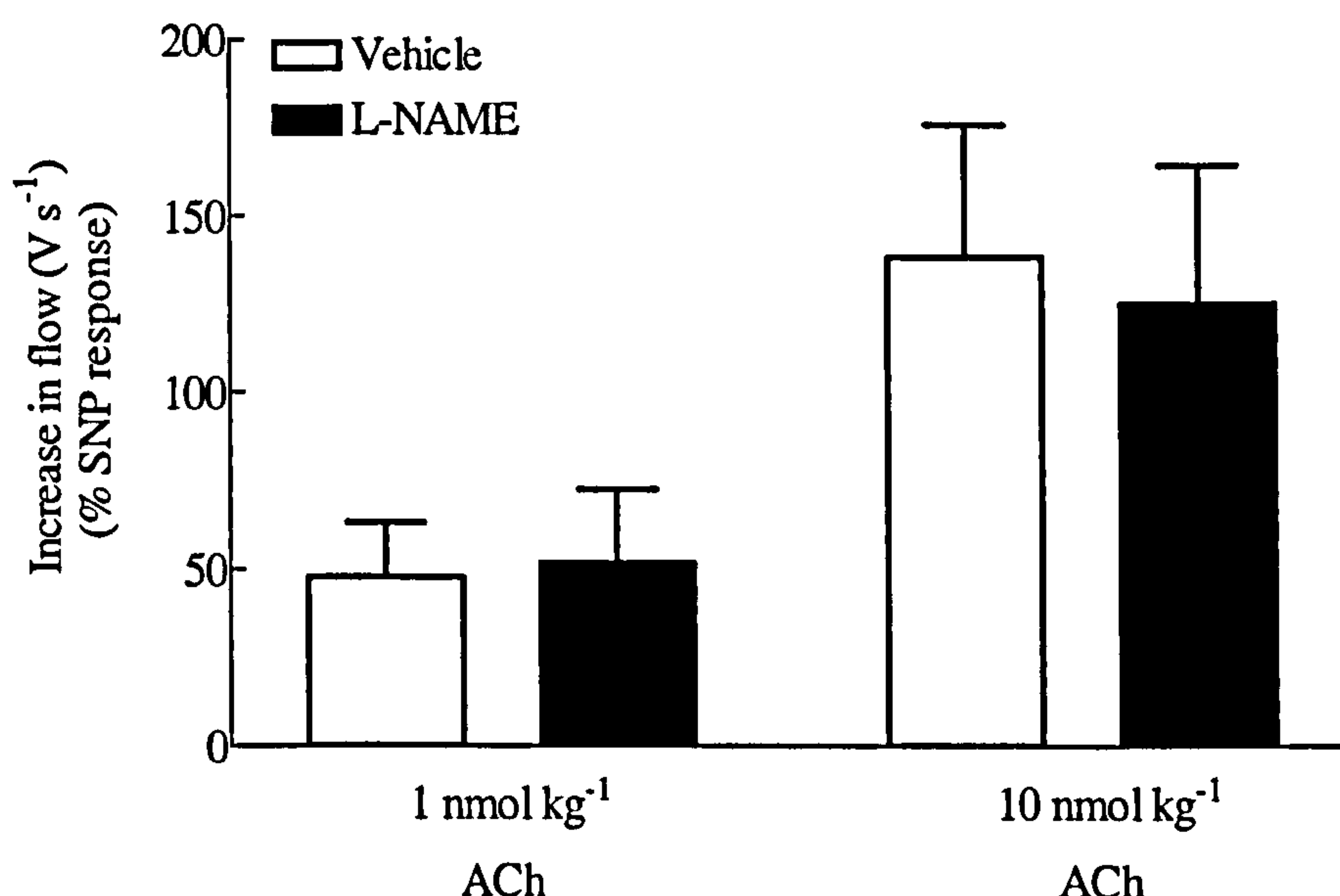


Figure 3.10 Acetylcholine (ACh, 1&10 nmol kg⁻¹ i.v.) -induced endothelium dependent changes in in ear arterial blood flow in vehicle (□) and L-NAME (100 mg kg⁻¹) (■) treated Wistar rats *in vivo*. Results are standardised with respect to vasodilation to sodium nitroprusside (SNP, 10 nmol kg⁻¹ i.v.). Data are expressed as mean±s.e.mean of 5-6 determinations.

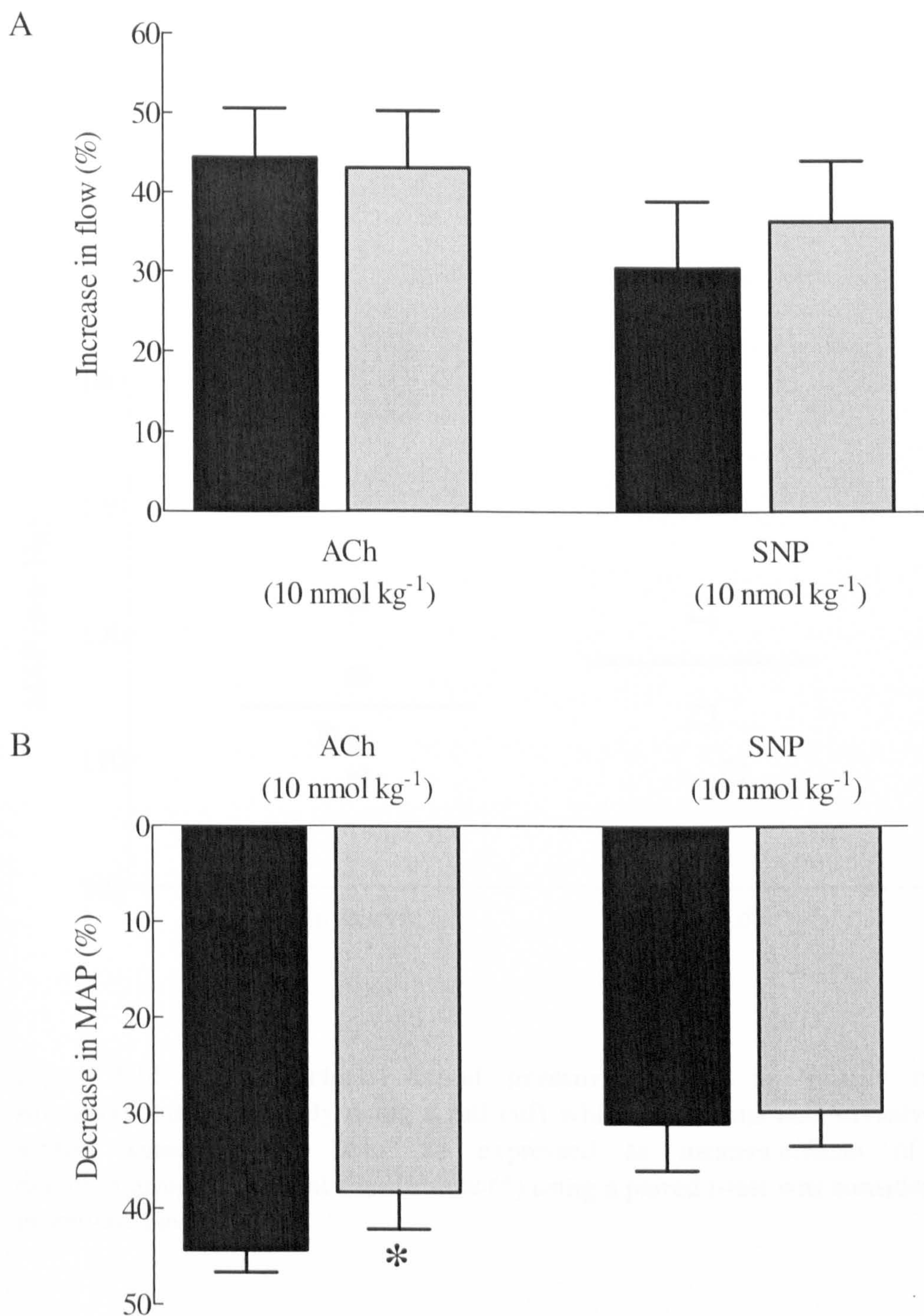


Figure 3.11 Acetylcholine (ACh, 10 nmol kg⁻¹ i.v.) and sodium nitroprusside (SNP, 10 nmol kg⁻¹ i.v) -induced changes in in ear arterial blood flow and MAP when administered via the femoral (■) or tail vein (■) in Wistar rats *in vivo*. Data are expressed as mean±s.e.mean of 6 determinations.

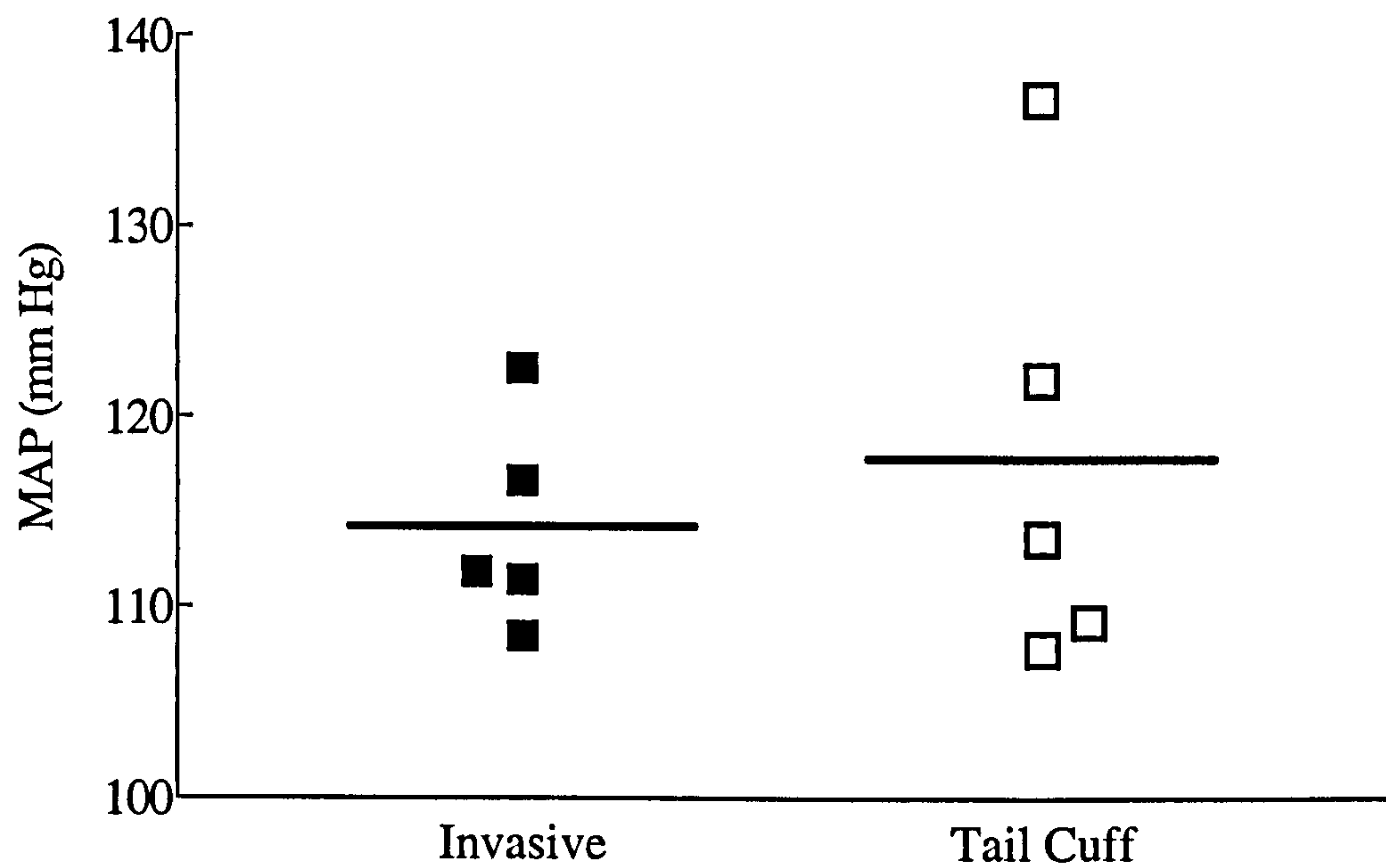


Figure 3.12 Mean arterial blood pressure (MAP) in Wistar rats measured non-invasively using a tail cuff whilst conscious and invasively whilst anaesthetised. Data are expressed as mean \pm s.e.mean of 5 determinations. Statistical significance (*) using a paired t-test was considered established at $P<0.05$.

3.4 DISCUSSION

Blood flow and pressure model. I have developed a model that enables simultaneous measurement of blood pressure and ear artery blood flow *in vivo* using laser Doppler fluxmetry. This model allowed correlation of agonist-induced changes in blood flow in a small artery with overall changes in blood pressure.

Initial studies showed the administration of endothelium-dependent and -independent vasodilators decreased MAP. This was followed by a simultaneous increase in pulsatile blood flow in the ear artery. The increase in flow was slightly time-delayed compared to the change in MAP and may reflect either the distribution time of the agonist to reach the sites of action, or autonomic control increasing blood flow in the ear artery as a consequence of decreased circulatory blood pressure. Another explanation for this time delay may be vascular tree variability where different receptors, endothelial mediators or signalling molecules are involved in vasodilation at various parts of the vasculature. Indeed, through the release of NO, organic nitrates are known to cause venous dilation and, at higher doses, arterial dilation. These variations in dilation demonstrate vasculature differences. Therefore the time delayed responses in my experiments are likely to be attributable to these differences.

In a previous study of the rat ear artery by Sanae & Hayashi (1998), theophylline, an adenosine antagonist, induced increases in blood pressure, heart rate and blood flow. L-NAME inhibited the blood flow without any changes in blood pressure and heart rate. They suggested the increased flow was due to an independent cardiac effect, influenced by adrenergic and nitrgenic pathways, as reserpine (a noradrenaline storage depleter) and propranolol (an adrenoceptor antagonist) reduced the vasodilation effects. This autoregulatory effect would be expected in the ear artery, since in the rat the ear is

important in the regulation of body temperature. However, increases in blood flow by ACh were not affected by reserpine, suggesting that the action of ACh was not through an autonomic mechanism in the ear.

My data also supports the notion of vascular tree variability and not autonomic control, as dose-dependent changes in blood flow were more sensitive to ACh than SNP, whilst changes in MAP were comparable. This is unlikely to be due to ACh stimulating the release of higher concentrations of endogenous NO, or that guanylyl cyclase levels are higher in the ear artery than in large conduit arteries. This is because L-NAME did not inhibit ACh-induced increases in ear arterial blood flow, but significantly impaired the decrease in MAP to ACh. Furthermore, SNP-induced changes in ear blood flow, MAP and HR were unaffected by L-NAME treatment, suggesting the L-NAME inhibition was specific to ACh-induced changes in MAP, as would be expected. Additionally, L-NAME infusion did not significantly change basal blood flow, MAP, or HR, thereby indicating ACh caused an agonist-stimulated endothelium-dependent increase in blood flow that was NO-independent. This notion is further supported by the fact that if in these experiments arteriole vasodilation was solely mediated through the action of NO, then decreasing MAP would correlate with increasing blood flow, equally for ACh and SNP. However, the results showed a significantly weaker correlation between MAP and blood flow with ACh ($r^2=0.71$) than with SNP ($r^2=0.88$) -induced vasodilation, suggesting ACh may involve another mediator as well as NO to provoke the vasodilating effect. This other mediator, is one that also causes enhanced blood flow in the ear artery by an endothelial-dependent mechanism, as SNP was less sensitive. These findings perhaps show that the NO-independent action of ACh is more important in the ear artery of the rat than it is systemically. In support of this, studies using iontophoretically applied ACh or SNP to the dermal circulation of men (Noon *et al.*, 1998) showed L-NAME had no effect, whereas aspirin inhibited ACh-induced vasodilation. However, L-NMMA inhibited the ACh-

stimulated decrease in blood flow in the brachial artery. The different changes observed in dermal and brachial artery blood flow support the notion of vascular tree variability. Other human brachial artery studies that support our data show ACh-stimulated increases in blood flow were inhibited by L-NMMA only at lower ACh concentrations (Bruning *et al.*, 1993). This suggests a second mechanism of cholinergic vasodilation at higher ACh concentrations, possibly through the release of a relaxing factor other than NO. In studies of the dermal circulation of healthy men, prostanoids were implicated in the ACh-stimulated endothelium-dependent NO-independent increase in blood flow, as aspirin inhibited ACh-induced vasodilation (Noon *et al.*, 1998). Further studies using a cyclo-oxygenase inhibitor such as indomethacin could be done to determine prostanoid involvement.

The involvement of a secondary mediator stimulated by ACh may be dependent on the types of muscarinic receptors present on the endothelium of the vascular tissue involved. It has been shown that the release of EDHF is mediated by M₁ receptors, and that NO is mediated by M₂ receptors (Komori & Suzuki, 1987; Keef & Bowen, 1989). Endothelium-dependent hyperpolarisations induced by ACh have been identified in various blood vessels (Taylor *et al.*, 1988; Chen *et al.*, 1988) and appear to contribute more to relaxation of small vessels than large arteries (Mugge *et al.*, 1991; Berman & Griffith, 1998; Feletou & Vanhoutte, 1999). This indicates a possible difference in the numbers of a particular receptor type in various blood vessels. Studies in the blood perfused rabbit hindquarter (Mugge *et al.*, 1991) showed L-NMMA increased vascular resistance but did not inhibit ACh vasodilation. This suggests that basal tone in the hindquarters resistance vessels may be NO mediated, but that the agonist-stimulated vasodilation may be mediated by another mechanism. More importantly, studies in the larger femoral arteries *in vitro* showed ACh-induced relaxation to be inhibited by L-NMMA. However, EDHF and NO may not be the only factors in the small versus large artery ACh-induced vasorelaxation. For example, a cytochrome P₄₅₀ metabolite of arachidonic acid may be responsible for L-NAME and

indomethacin resistant changes induced by ACh in the diameter of large epicardial coronary arteries in dogs (Ming *et al.*, 1997). Though, the precise role of endothelium-derived relaxing factors such as, NO, prostacyclin and hyperpolarising factor(s) in this hyper-reactivity, remains to be established.

It remains possible that ACh may have a direct action on vascular smooth muscle, or SNP may not be as able to access the smooth muscle as efficiently as agonist-stimulated NO, released to the sub-luminal space. This is unlikely given that SNP was not more effective than ACh at increasing blood flow in the smaller vessels where the endothelium is fenestrated; a structure which allows for greater diffusion across the endothelial membrane. Either way, our study shows a novel coexistence of both a NO-mediated and NO-independent action of ACh regulating blood flow in the rat ear artery *in vivo*; a finding that is contrary to previous results obtained by photoplethysmography in the rabbit ear artery where ACh-induced changes in both MAP and flow that were NO-dependent. However, our results in the rat are consistent with an involvement of EDHF, and similar effects have been shown *in vitro* in smaller arterioles (Berman & Griffith, 1998).

Since any potential non-invasive method of endothelial function measurement would require drug administration via the tail vein, we compared tail with jugular vein drug administration. Both routes provoked essentially the same increase in arterial blood flow, although ACh-induced decreases in MAP were reduced when administered via the tail vein compared to jugular vein, perhaps because the tail vein is further down the venous circulatory system. Overall, this system may be a suitable method in which to non-invasively assess endothelial function in small arteries and arterioles in the rat where repeat measurements of vascular/endothelial activity in the same animal are desirable over an extended period of time.

The second method analysed was the indirect rat tail blood pressure monitoring. Results show that the indirect tail cuff measurement was 117.85 ± 5.3 mm Hg ($n=5$) and direct invasive MAP measurement was 114.23 ± 2.5 mm Hg ($n=5$) whilst the same rats were anaesthetised. These data are not significantly different, suggesting that basal endothelial function may not be altered by anaesthesia with thiopentone (at least in our hands); although induction of anaesthesia with intravenous thiopental is often accompanied by hypotension. Studies have shown that thiopentone inhibits endothelium-dependent relaxation and nitrite production in aortic rings, indicating that thiopentone inhibits NOS (Castillo *et al.*, 1999). Further evidence has been shown *in vitro* where activity of the enzyme iNOS was decreased by thiopentone (Galley & Webster, 1996). Interestingly, there was no correlation between the anaesthetised and conscious rat blood pressure, suggesting that the small difference between groups was arbitrary and was probably due to minor differences in animal variation and handling. Unfortunately, this model did not provide reproducible data blood pressure data from the obese Zucker rat. Indeed, a similar instrument, the photoelectric pulse sensor, has been refused registration with the FDA because it cannot be calibrated for skin pigmentation, skin thickness, etc (Stoelting Co, USA). This is of particular relevance in the Zucker rat as it has brown skin.

Perfused Hindquarters. A principal aim of this project was to develop a method to study endothelial function in an insulin sensitive tissue that provides a large resistance vascular area – the rat hindquarters. In initial validation studies, a fall in effluent outflow over time was seen. This was probably due to development of oedema that became increasingly evident after 90 min. Such changes may be due to vasodilation in the perfused hindquarters causing a decrease in vascular resistance, resulting in either a decrease in blood pressure and/or flow rate. However, this haemodynamic relationship also applies when water from the PSS accumulates in the interstitial compartment, contributing to oedema formation and an increase in vascular resistance. Oedema is known to contribute to high blood pressure

and vascular resistance in patients with cardiovascular disease. Oedema development was expected in my experiments as the PSS did not contain colloidal material necessary to maintain the osmotic balance. However, it is considered acceptable to carry out perfusion experiments for an optimal period or to decrease the perfusion flow rate (Guarner *et al.*, 1993) to avoid oedema. In my studies, the hindquarters basal perfusion pressure was stable for 90 min, suggesting that vascular resistance was not compromised. Reproducible vascular reactivity data was obtained between 60 and 120 min after the start of perfusion, therefore all our experiments were carried out within 90 min.

In vasodilation studies, vascular reactivity is assessed using the endothelium-dependent NO and endothelium-independent dilators, ACh and SNP respectively. Both agonist-stimulated endothelium-derived and exogenous NO were effective in the reversal of established vascular contractile tone. When basal NO synthesis was inhibited by L-NAME, a leftward shift in the concentration response curve to Phe was observed and contractile tone was enhanced, suggesting NO had antagonised the Phe-induced contraction. Similarly, contractile tone was more effectively reversed by exogenous NO (SNP) in L-NAME treated preparations than in control preparations. However, agonist-stimulated endothelial-dependent vasorelaxation partially reversed the enhanced contractile tone in the presence of L-NAME. This may be because L-NAME had not completely inhibited NO synthesis. Previous reports have shown L-NAME produces almost 100 % inhibition of ACh-stimulated relaxation *in vitro*, but only approximately 75 % inhibition *in vivo* (Rees *et al.*, 1990). Therefore a partial vasorelaxation would be expected in an *in situ* model. Furthermore, this partial endothelium-dependent and enhanced endothelium-independent vasodilation may be greater in the absence of basal NO, as guanylyl cyclase would be under-stimulated which would lead to super-sensitivity. Super-sensitivity has been established in studies where the endothelium was removed (Moncada *et al.*, 1991).

The *in situ* rat perfused hindquarters is thus an important preparation for the investigation of endothelial function in a pre-diabetic model of oxidant stress, such as the obese Zucker rat (see later), since it facilitates the assessment of endothelium-dependent vasodilator function in a physiological relevant vascular bed. It is important, however, to compare endothelial function in this uncomplicated preparation to that of a more complex *in vivo* model.

Chapter 4

*Investigation of insulin resistance,
endothelial reactivity and oxidant stress
in the obese Zucker rat*

4.1 INTRODUCTION

The WHO states that there is no generally accepted definition for the type 2 diabetes metabolic syndrome. However in working terms, it is the combination of hypertension, dyslipidaemia, insulin resistance, hyperinsulinaemia, glucose intolerance, and obesity, (particularly central obesity). Metabolic syndrome is an early stage of diabetes that indicates the individual is predisposed and is at high risk for diabetes; although changes in diet and exercise has been shown to slow the onset of disease in animal models (Becker-Zimmermann *et al.*, 1982; Reaven *et al.*, 1988).

The Zucker rat (*fa/fa*) is an obese model of pre-diabetes – metabolic syndrome X, and is in many aspects a suitable model to investigate the relationship between metabolic syndrome, insulin resistance and diabetes. In 1985 Ionescu *et al* demonstrated that, at 13-14 weeks-old, obese Zucker rats had significantly higher basal glucose and insulin (10 fold) levels, and impaired glucose disposal compared to lean rats. In younger 6-7 week-old obese rats, levels of glucose were normal and insulin moderate (3 fold higher) compared to the lean rats; glucose disposal too was impaired compared to 6-7 week-old lean littermates but to a lesser extent than that seen in the 13 week-old rats. Not all investigators have found hyperglycaemia (Triscari *et al.*, 1979). This may be due to a compensatory mechanism for insulin resistance that causes an increased β -cell secretion of insulin, thereby preventing hyperglycaemia (a notion structurally indicated by hypertrophic and hyperplastic β -cells (Ionescu *et al.*, 1985). This is similar to most obese humans (Kraegen *et al.*, 2001), and is therefore an important pre-diabetic model that can be used to study the early stages of insulin resistance prior to the development of frank diabetes. The additional components of the metabolic syndrome have also been investigated in the obese Zucker rat, and show raised triglycerides (Boulangue *et al.*, 1981), hyperlipidaemia, microalbuminuria and raised arterial blood pressure (Kasiske *et al.*, 1985).

The initial hypothesis was that endothelial dysfunction, insulin resistance and oxidant stress would coexist in the 12 week-old Zucker rat, and these factors would contribute to the development of the metabolic syndrome and then to diabetes and its complications. The relationship between these pathologies, and how each could enhance the disease process of the other has not been determined. It may be that defects in insulin signalling contribute to the increased endothelial dysfunction and insulin resistance (Baron, 2002). Alternatively, increased levels of ROS (with or without the above pathologies) react with NO and hence reduce basal and induced endothelial vasodilation. This impaired endothelial function, and its resultant decreased vasodilation, can contribute to the decreased post prandial glucose disposal. Interestingly, it has been demonstrated that, in individuals with type 2 diabetes, post prandial glucose levels may be better predictors of glycaemic control than fasting plasma glucose (Avignon *et al.*, 1997). It has been estimated that most exposure to excess glucose occurs in post prandial periods. Many epidemiological studies suggest that post-prandial hyperglycaemia, even in the absence of fasting hyperglycaemia, is an independent risk factor for the development of macro- and microvascular complications associated with diabetes (Curb *et al.*, 1995; Lowe *et al.*, 1997). This was probably due, at least in part, to hyperglycaemia causing non-enzymatic protein glycation. The relative contribution of post-prandial and fasting glucose to protein glycation is, however, not known. Some studies have shown enhanced lipid peroxidation associated with post-prandial hyperglycaemia (Ceriello *et al.*, 1998). This may be due to autooxidation of glucose, AGE-formation, glycation of enzymatic antioxidants and/or stimulation of the polyol pathway. It may be that by normalizing post-prandial glucose and the oxidative load, the ensuing diabetic complications can be decreased.

Endothelial function, oxidant stress and insulin sensitivity were assessed in the same age matched obese group. Studies of endothelial function in the obese Zucker rat have generated conflicting results in some cases, showing both increases and decreases in

endothelial function. Supporting findings of increased endothelial function studies, have shown that obese Zucker rats have exaggerated intracellular Ca^{2+} responses to vasoactive agents (Abel & Zemel, 1993). This difference may simply be age related. Alternatively it may also be an indication of the stage of the disease progression, as Sexl *et al* (1995) showed enhanced endothelial function in 12 week obese rats and normal function in 24 and 36 week-old obese rats. Other studies, however, have shown NOS activity in skeletal muscle samples to be greater in lean than in obese rats (Young & Leighton, 1998), unfortunately the age of the rats was not stated.

In some of these studies, endothelial function measurements had focused on aorta and other arterial vessels from the obese Zucker rat. The insulin sensitive hindquarters perfusion system would, however, be more suitable model to understand a relationship between insulin resistance and endothelial function. This is because it is an insulin sensitive tissue and also because the microcirculation contains the greatest amount of the total animal blood volume. Therefore changes in pulse pressure can have significant effects on endothelial cell permeability and the redistribution of blood plasma into adjacent fluid compartments in the skeletal muscle. This can lead to alterations in the transport of substrates and waste products between blood and cells, and may therefore be important in the uptake of glucose in these insulin sensitive tissues. Studies would also concentrate on the involvement of NO in obese Zucker rat endothelial function in perfused hindquarters. The use of L-NAME in the functional studies can then be compared to the indirect biochemical evidence for NOS activity using the Griess method. The Griess method measures nitrite/nitrate – metabolites of NO that are stable in the plasma before their excretion in the urine.

Oxidative stress has been suggested as a significant contributing factor in the development of endothelial dysfunction and insulin resistance (Nourooz-Zadeh *et al*, 1997; Perticone *et al*, 2001). Elevated levels of 8-epi-PGF_{2α} have been detected in type 2 diabetic patients

(Gopaul *et al.*, 1995), implying increased levels of ROS. Other studies have shown a doubling in plasma lipid peroxides in type 2 diabetic subjects compared to control (Nourooz-Zadeh *et al.*, 1997), and a reduction in total plasma antioxidant capacity (Ceriello *et al.*, 1997). Indeed, increased oxidant stress has been shown in obese Zucker rats – in liver studies, obese Zucker rat livers contained significantly less GSH, tocopherol, and catalase, compared to leans. In addition, pre-treatment with vitamins C and E completely corrected the decreases in GSH, tocopherol, and catalase (Soltys *et al.*, 2001). Other studies have shown vascular $O_2^{\cdot-}$ levels were elevated in obese compared to the lean rats, and that functionally, treatment of cremaster muscles of obese rats with the $O_2^{\cdot-}$ scavengers can improve arteriolar dilation to ACh and SNP (Frisbee & Stepp, 2001).

Oxidative stress in the Zucker rat was assessed by measurement of 8-epi-PGF_{2α}, and TAOS in the rat plasma (Laight *et al.*, 1999A; 1999B). 8-epi-PGF_{2α}, is the most studied F₂-isoprostane and is present in human plasma, urine (Morrow *et al.*, 1999), tissues and endothelial cells (Gopaul *et al.*, 1994). 8-epi-PGF_{2α}, is a specific marker of lipid peroxidation, whereas TAOS measurements incorporate the antioxidant status of many plasma solutes such as ascorbate, glutathione, urate and albumin. TAOS is measured from plasma samples and it estimates the protective anti-oxidative ability present in the plasma. The plasma contains free radical scavengers such as vitamin C and E which can be rapidly consumed while scavenging ROS. In addition to scavenging ROS, plasma can reduce oxidised forms of antioxidants back to the active form. For example, GSH regenerates vitamin C, which, in turn, regenerates vitamin E. It follows that the total plasma antioxidant capacity is the result of interactions between many different compounds. Interestingly, 8-epi-PGF_{2α}, is a potent vasoconstrictor in the kidney (Fukunaga *et al.*, 1993), lungs (Banerjee *et al.*, 1992; Kang *et al.*, 1993), coronary (Kromer & Tippins, 1996) and retinal (Lahaie *et al.*, 1998) vessels at nanomolar concentrations. It was therefore appropriate to test for vasoconstrictor effect in our vascular preparations – hindquarters and aortic rings.

Plasma levels of 8-epi-PGF_{2α} are increased in type 2 diabetic patients (Gopaul *et al.*, 1995). However, whether 8-epi-PGF_{2α} also contributes to endothelial dysfunction is not presently known.

4.2 RESULTS

4.2.1 Insulin resistance in the obese Zucker rat

High plasma insulin in obese Zucker rats. The basal plasma insulin levels in the obese (5.93 ± 1.4 ng mL⁻¹, $n=6$) were significantly higher than that of the lean (0.46 ± 0.09 ng mL⁻¹, $n=5$; $P<0.05$) Zucker rat.

After bolus glucose (0.5 g kg⁻¹ i.v.) administration, plasma insulin was increased in the obese (AUC was 221.05 ± 20.5 ng mL⁻¹ min⁻¹, $n=4$) compared to lean (AUC was 46.71 ± 7.5 ng mL⁻¹ min⁻¹, $n=5$; $P<0.05$) Zucker rats, and remained higher in the obese for the duration of the timecourse (24 min) (Fig. 4.1A).

High plasma glucose in obese Zucker rats. Basal plasma glucose levels in the obese (5.15 ± 0.6 mM, $n=7$) were higher than those of the lean (3.09 ± 0.2 mM, $n=7$; $P<0.05$) Zucker rat.

After glucose (0.5 g kg⁻¹ i.v.) administration during the glucose tolerance test, plasma glucose was increased in the obese (AUC was 297.23 ± 19.38 mM min⁻¹, $n=7$) compared to lean (AUC was 222.69 ± 8.1 mM min⁻¹, $n=7$; $P<0.05$) Zucker rats, and remained higher in the obese for the duration of the timecourse (24 min) (Fig. 4.1B).

4.2.2 Endothelial reactivity in the Zucker rat perfused hindquarters

Mean body weight was greater in 13 week-old obese (413.6 ± 15.2 g, $n=5$) relative to lean animals (280.2 ± 4.2 g, $n=5$; $P<0.05$). Basal perfusion pressure in obese preparations (28.5 ± 6.1 mm Hg, $n=5$) was comparable with that of lean preparations (38.1 ± 5.8 mm Hg, $n=5$). Similarly, Phe-elevated perfusion pressure in obese preparations (144.2 ± 20.5 mm Hg,

$n=5$) did not differ from that of lean Zucker rat preparations (129.6 ± 6.0 mm Hg, $n=5$; $P>0.05$).

Enhanced endothelial vasodilator function in obese Zucker rats. Vasodilator responses to ACh (0.0001-10 nmol) were enhanced ($P<0.05$) in obese relative to lean animals (Fig. 4.2; table 4.1), while maximal vasodilation to SNP (100 nmol) was similar (obese: 59.6 ± 9.8 %, $n=5$; lean 51.9 ± 2.6 %, $n=5$; $P>0.05$). When responses to ACh (0.0001-10 nmol) were standardised with respect to SNP (100 nmol) as an assessment of hindquarters endothelial function, the data still showed a significantly greater vasodilation in the obese relative to lean animals.

When assessed as pD_2 (i.e. the negative \log_{10} of the concentration eliciting the half-maximal response), the obese ($pD_2=11.03 \pm 0.19$, $n=5$) showed a slight (3-fold) increase in the sensitivity of vasodilation to ACh relative to lean ($pD_2=10.53 \pm 0.13$, $n=5$; $P=0.05$) preparations. Similarly, when responses to ACh (0.0001-10 nmol) were standardised with respect to SNP (100 nmol), the pD_2 values were the same to two decimal places.

L-NAME inhibited ACh-induced vasodilation. Basal perfusion pressure in control preparations (lean: 27.9 ± 3.2 ; obese: 29.0 ± 1.4 mm Hg, $n=5$) was comparable with that of L-NAME treated preparations (lean: 24.7 ± 1.8 ; obese: 27.0 ± 2.3 mm Hg, $n=5$; $P>0.05$). Similarly, Phe elevated perfusion pressure was comparable in L-NAME (lean: 153.0 ± 32.6 ; obese: 174.0 ± 32.1 mm Hg, $n=5$; $P>0.05$) relative to control preparations (lean: 155.3 ± 8.1 ; obese: 157.3 ± 8.6 mm Hg, $n=6$). The ACh (0.0001-10 nmol) -induced vasodilation was significantly greater in the obese compared to the lean. Additionally, this enhanced vasodilation in the obese was maintained with the administration of L-NAME (2.1 μ mol), which impaired maximal vasodilation in both lean and obese Zucker rats ($P<0.05$) (Fig. 4.3).

	OBESE		LEAN	
Treatment	pD_2	E_{\max}	pD_2	E_{\max}
Control	10.88+0.06*	131.06+13.9	10.51+0.04	102.4+16.6
NAME	10.42+0.05*†	75.1+5.9*†	10.20+0.06†	46.7+8.4†

Table 4.1 Vasorelaxation to ACh (0.0001-10 μ M) in Phe pre-contracted obese and lean Zucker rat perfused hindquarters in the presence and absence of L-NAME (2.1 μ mol). E_{\max} was reached at 1 μ M. Data are expressed as mean \pm s.e.mean of 4-5 determinations. * P <0.05 with respect to corresponding treatment in the lean group; † P <0.05 with respect to corresponding control group.

High blood pressure in obese Zucker rats. The mean arterial blood pressure was raised in 13 week-old obese (136.0 \pm 3.6 mm Hg, n =8) compared to lean (116.4 \pm 6.7 mm Hg, n =8; P <0.05) Zucker rats (Fig. 4.4). In this group body weight was greater in obese (477.88 \pm 13.2 g, n =8) relative to lean animals (298.9 \pm 8.4 g, n =8).

Low blood volume in obese Zucker rats. The administration of Evans blue caused a small, non-significant rise in obese MAP (1.7 \pm 1.6mm Hg, n =8) and decrease in lean MAP (-1.4 \pm 1.6 mm Hg, n =8). Evans blue obeys Beer Lambert law between 1.5-25 mg L⁻¹. The slope of the standard curve (0.5-32 mg L⁻¹) constructed was linear (r^2 = 0.99, slope = 0.5726 \pm 0.001 mg L⁻¹ cm abs⁻¹).

The haematocrit, or packed cell volumes were comparable in the obese (44.09 \pm 1.07, n =8) and lean (46.5 \pm 0.99, n =8; P >0.05) Zucker rats. There was a correlation between weight and blood volume in both obese (r^2 =0.85) and lean (r^2 =0.75). Interestingly, the results showed a decreased blood volume per 100 g body weight in the obese (4.2 \pm 0.10 cm³ 100 g⁻¹, n =8) Zucker rat compared to the lean (6.0 \pm 0.15 cm³ 100 g⁻¹, n =8; P <0.05) (Fig. 4.5).

High plasma nitrate/nitrite levels in obese Zucker rats. Plasma nitrate/nitrite levels in the obese ($33.39 \pm 2.6 \mu\text{M}$, $n=3$) were higher than that of the lean ($20.93 \pm 1.9 \mu\text{M}$, $n=6$; $P<0.05$) Zucker rat (Fig. 4.6).

4.2.3 Oxidant stress in the obese Zucker rat

High plasma 8-epi-PGF_{2α} levels in obese Zucker rats. Plasma 8-epi-PGF_{2α} levels in the obese ($3.7 \pm 0.4 \text{ ng mL}^{-1}$, $n=5$) were higher than that of the lean ($0.7 \pm 0.2 \text{ ng mL}^{-1}$, $n=5$; $P<0.05$) Zucker rat (Fig. 4.7).

High plasma TAOS levels in obese Zucker rats. Plasma TAOS levels in the obese ($46.85 \pm 1.1 \text{ ng mL}^{-1}$, $n=4$) were higher than that of the lean ($42.67 \pm 0.4 \text{ ng mL}^{-1}$, $n=6$; $P<0.05$) Zucker rat (Fig. 4.8).

Vasoconstriction to 8-epi-PGF_{2α} in the rat perfused hindquarters. Cumulative infusions of 8-epi-PGF_{2α} (0.1-3000 nmol) in the presence of L-NAME (2.1 μmol) elicited concentration-dependent, sustained increases in perfusion pressure ($pD_2=5.6 \pm 0.77$, $n=3$) (Fig. 4.9A). In the absence of L-NAME, 8-epi-PGF_{2α} did not produce measurable contractile responses.

Vasoconstriction to 8-epi-PGF_{2α} in rat aortic rings. 8-epi-PGF_{2α} (0.1-3000 nmol) elicited concentration-dependent and sustained increases in tension. When assessed as pD_2 values, the L-NAME ($pD_2=2.8 \pm 0.15$, $n=4$) cumulative concentration curve was enhanced compared to the control ($pD_2=3.6 \pm 0.10$, $n=4$; $P<0.05$) (Fig. 4.9B).

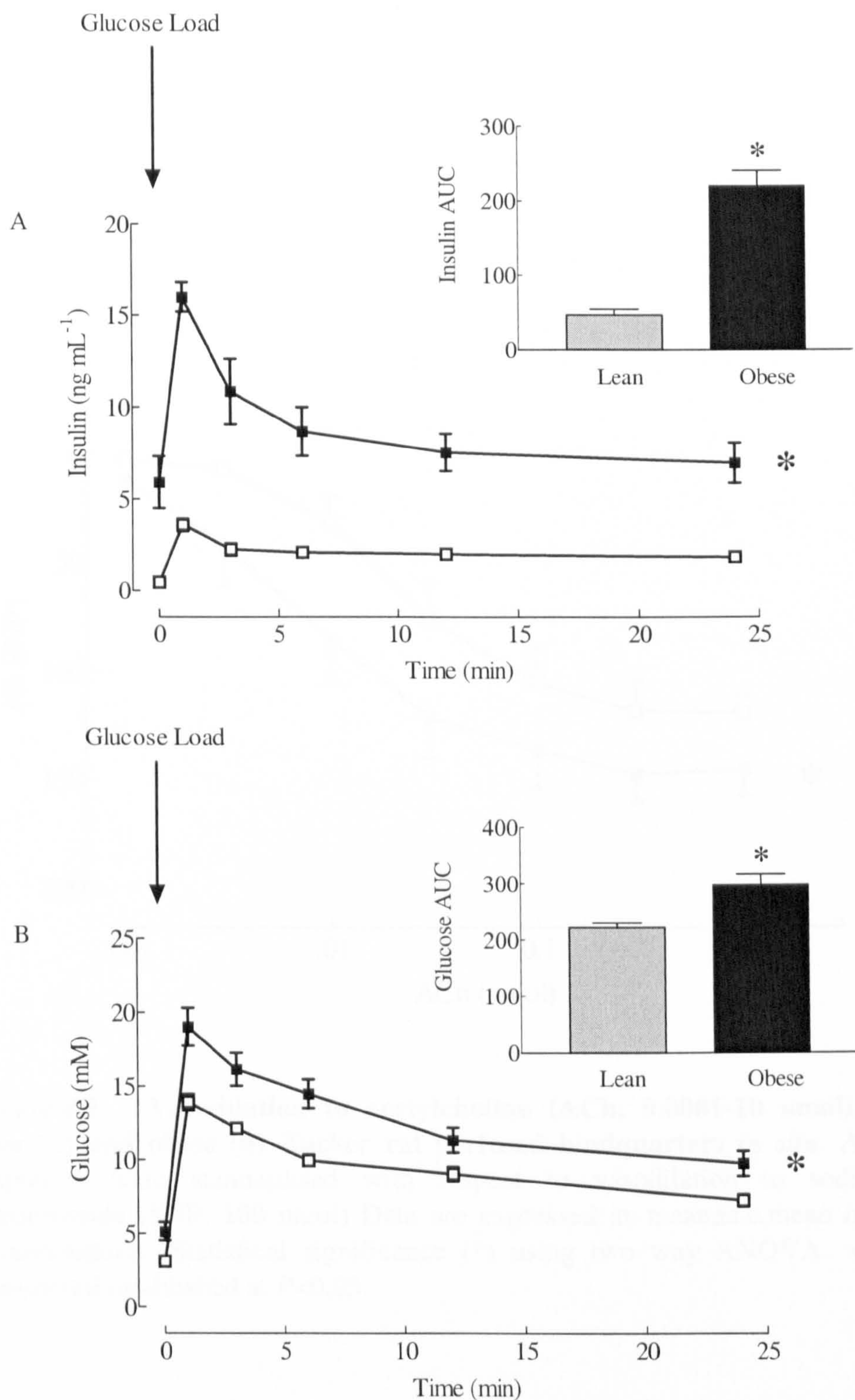


Figure 4.1 Glucose tolerance test (g.t.t) in 13 week-old lean (□) and obese (■) Zucker rats A. Comparison of plasma insulin (AUC, 0-24 min). B. Plasma glucose (AUC, 0- 24 min). Data are expressed as mean±s.e.mean of 4-7 determinations. Statistical significance lean vs. obese (*) was considered established at $P < 0.05$ using a students t-test.

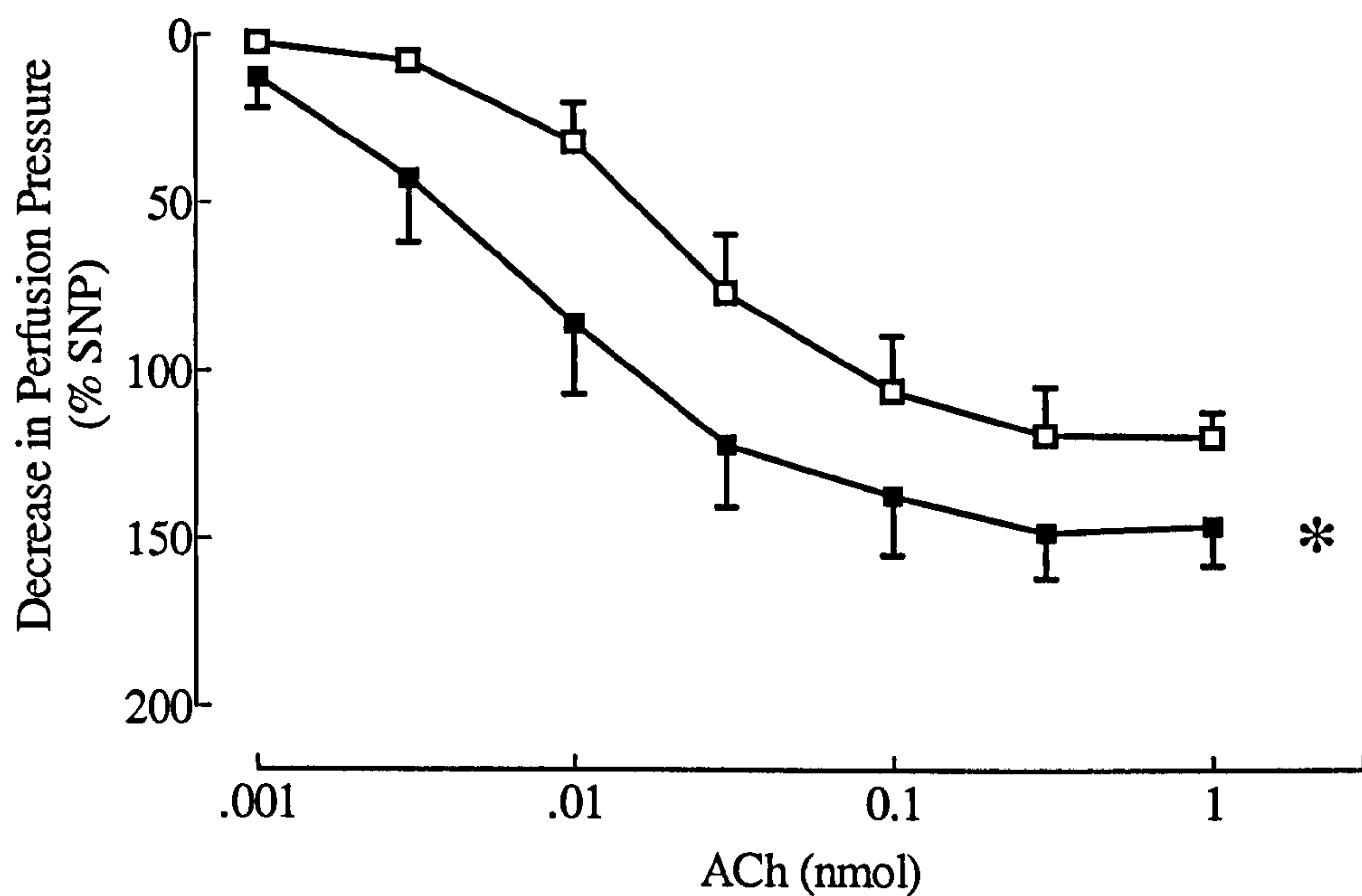


Figure 4.2 Vasodilation to acetylcholine (ACh, 0.0001-10 nmol) in lean (\square) and obese (\blacksquare) Zucker rat perfused hindquarters *in situ*. ACh responses were standardised with respect to vasodilation to sodium nitroprusside (SNP, 100 nmol) Data are expressed as mean \pm s.e.mean of 5 determinations. Statistical significance (*) using two way ANOVA was considered established at $P<0.05$.

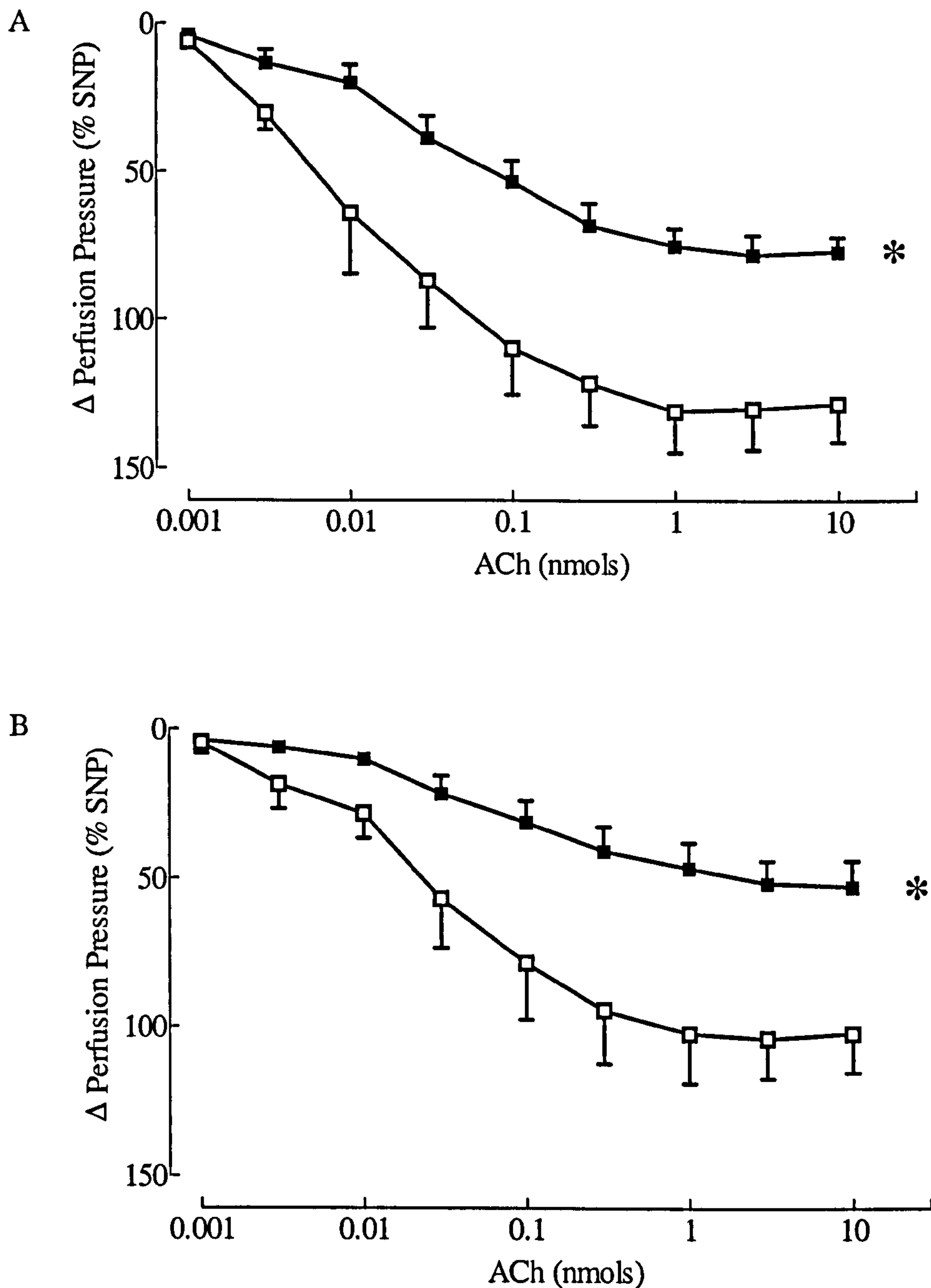


Figure 4.3 Vasodilation to acetylcholine (ACh, 0.0001-10 nmol) was assessed in phenylephrine (100 nmol) pre-constricted Zucker rat perfused hindquarters *in situ*. Comparison of A. Obese B. Lean control (□) with L-NAME (■) treated preparations. ACh responses were standardised with respect to sodium nitroprusside (SNP, 100 nmol) Data are expressed as mean±s.e.mean of 4-5 determinations. Statistical significance (*) using two way ANOVA was considered established at $P<0.05$.

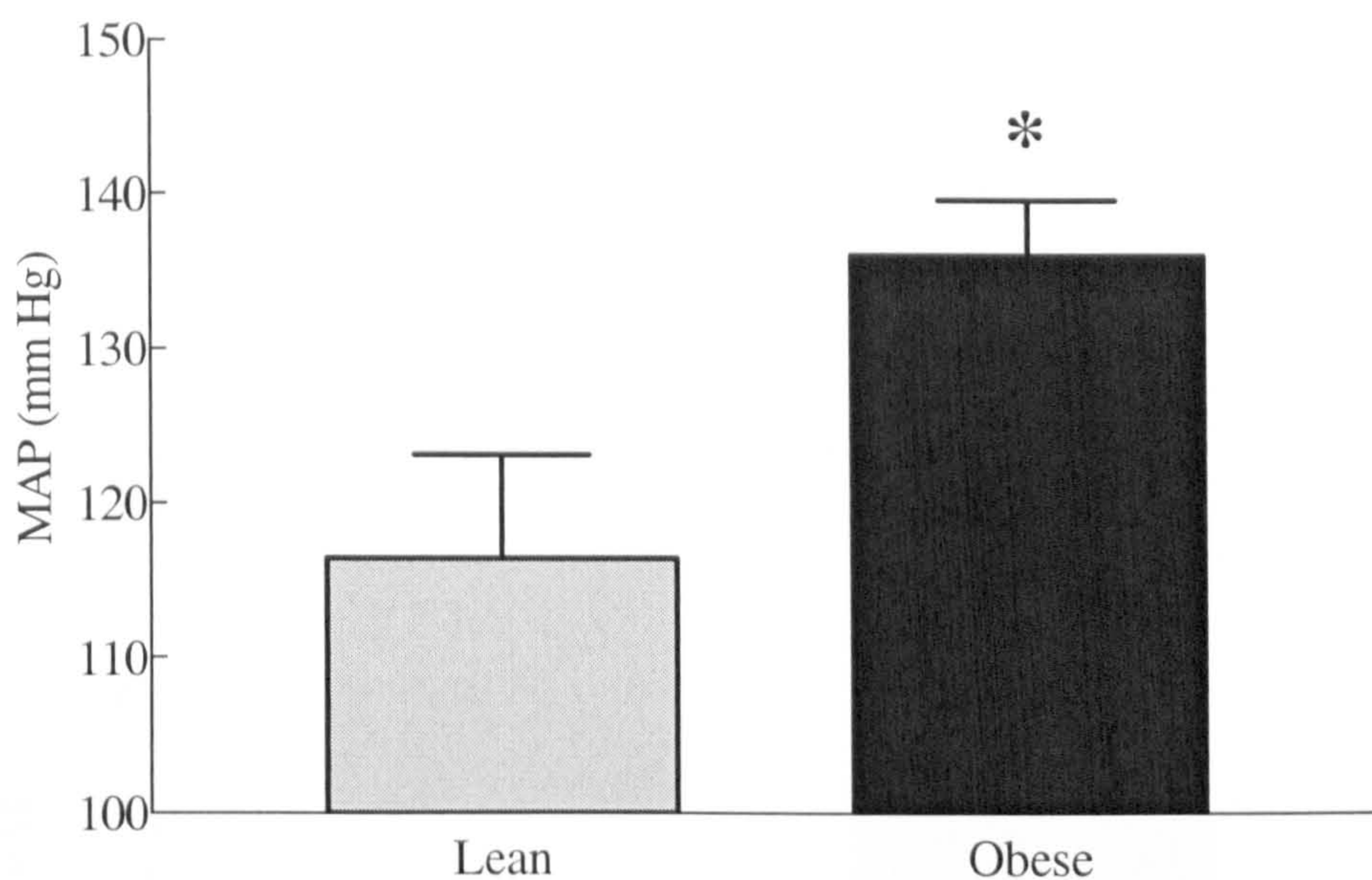


Figure 4.4 Mean arterial blood pressure (MAP) in anaesthetised 13 week-old lean and obese Zucker rats. Data are expressed as mean \pm s.e.mean of 8 determinations. Statistical significance (*) using an unpaired student t-test was considered established at $P<0.05$.

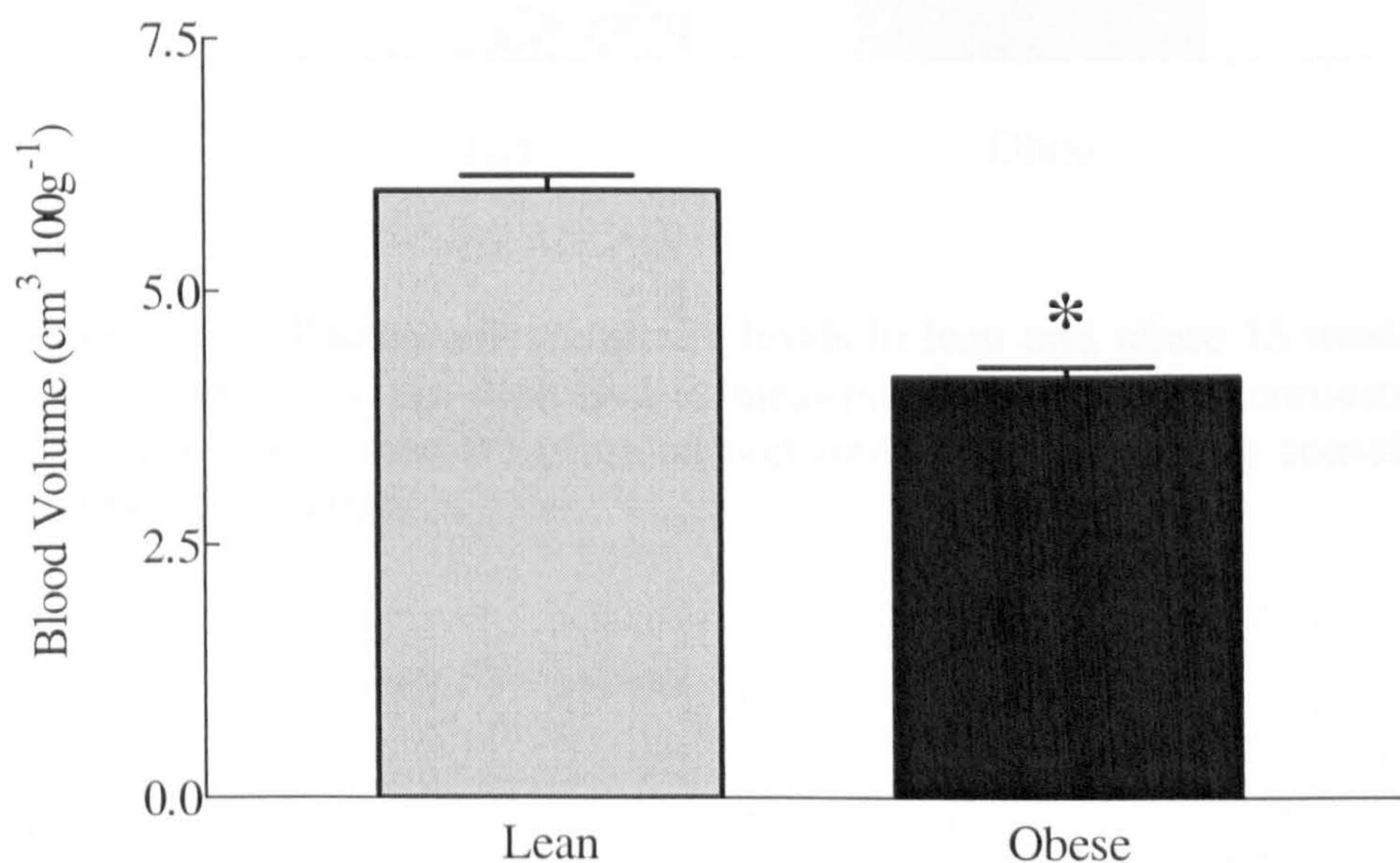


Figure 4.5 Assessment of blood volumes in lean and obese 13 week-old Zucker rats. Data are expressed as mean \pm s.e.mean of 8 determinations. Statistical significance (*) using an unpaired student t-test was considered established at $P<0.05$.

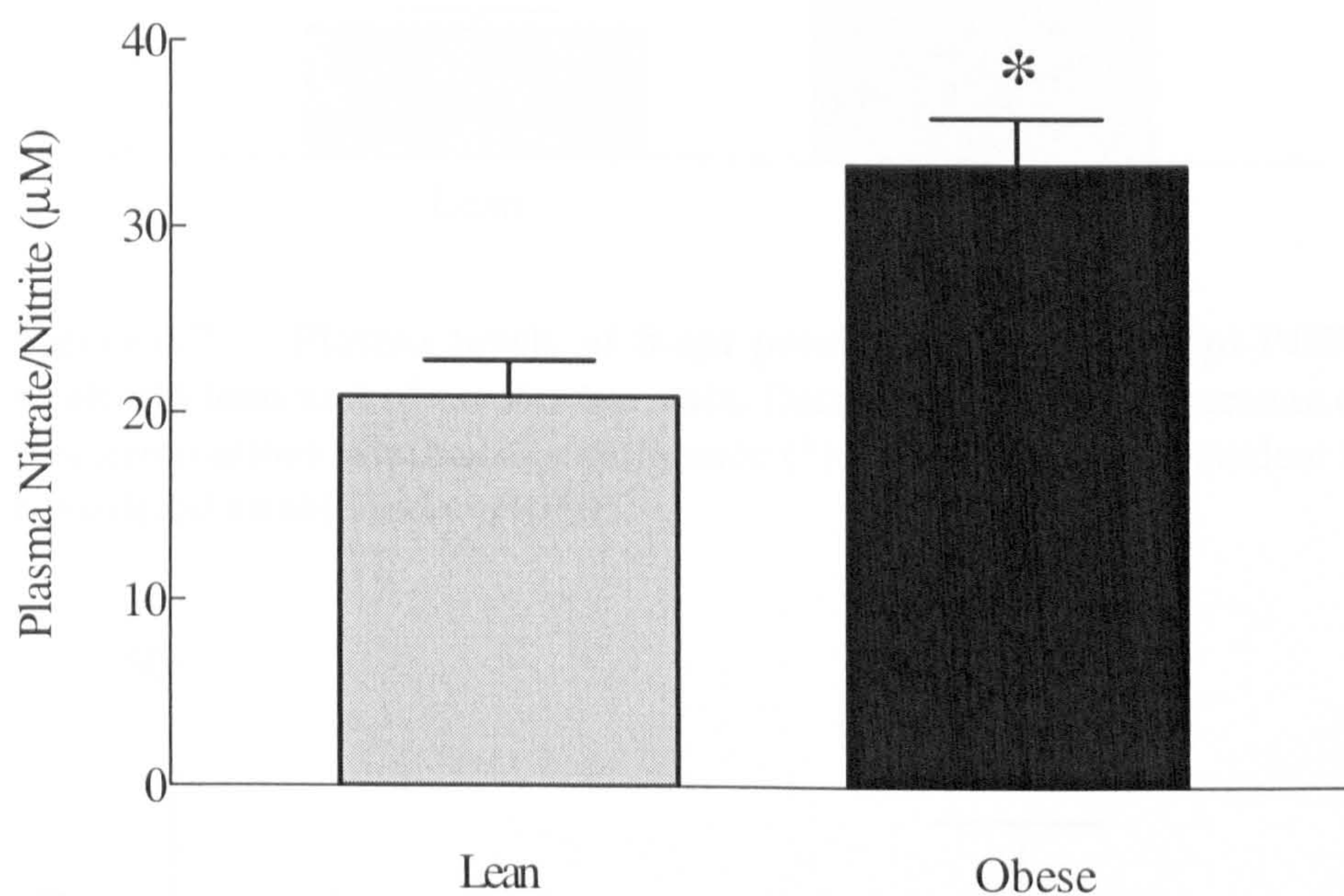


Figure 4.6 Plasma nitrate/nitrite levels in lean and obese 13 week-old Zucker rats. Data are expressed as mean+s.e.mean of 3-6 determinations. Statistical significance (*) using an unpaired student t-test was considered established at $P<0.05$.

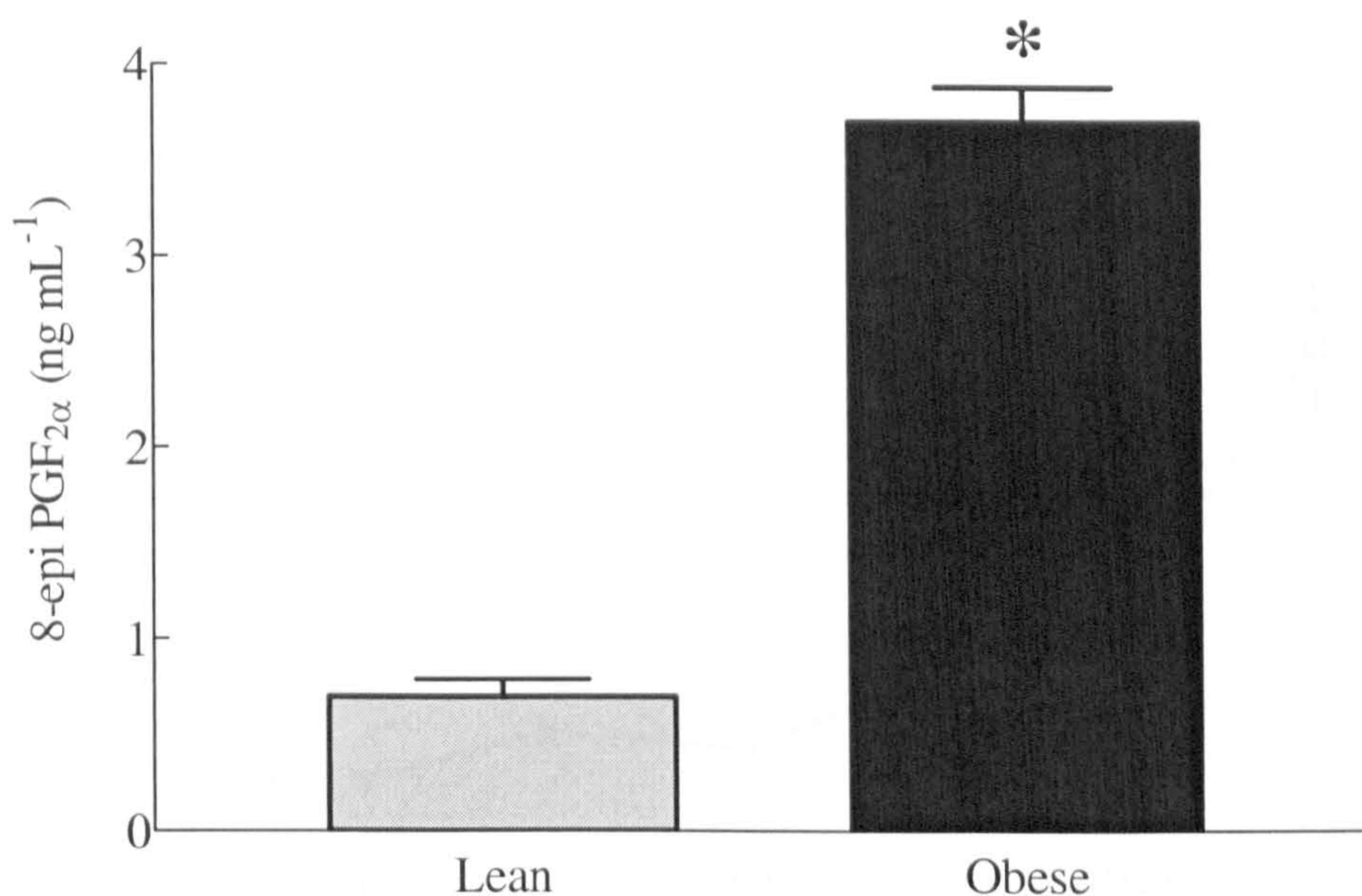


Figure 4.7 Plasma levels of 8-epi prostaglandin F_{2α} (8-epi-PGF_{2α}) in 13 week-old lean and obese Zucker rats. Data are expressed as mean±s.e.mean of 5 determinations. Statistical significance (*) using an unpaired student t-test was considered established at $P<0.05$.

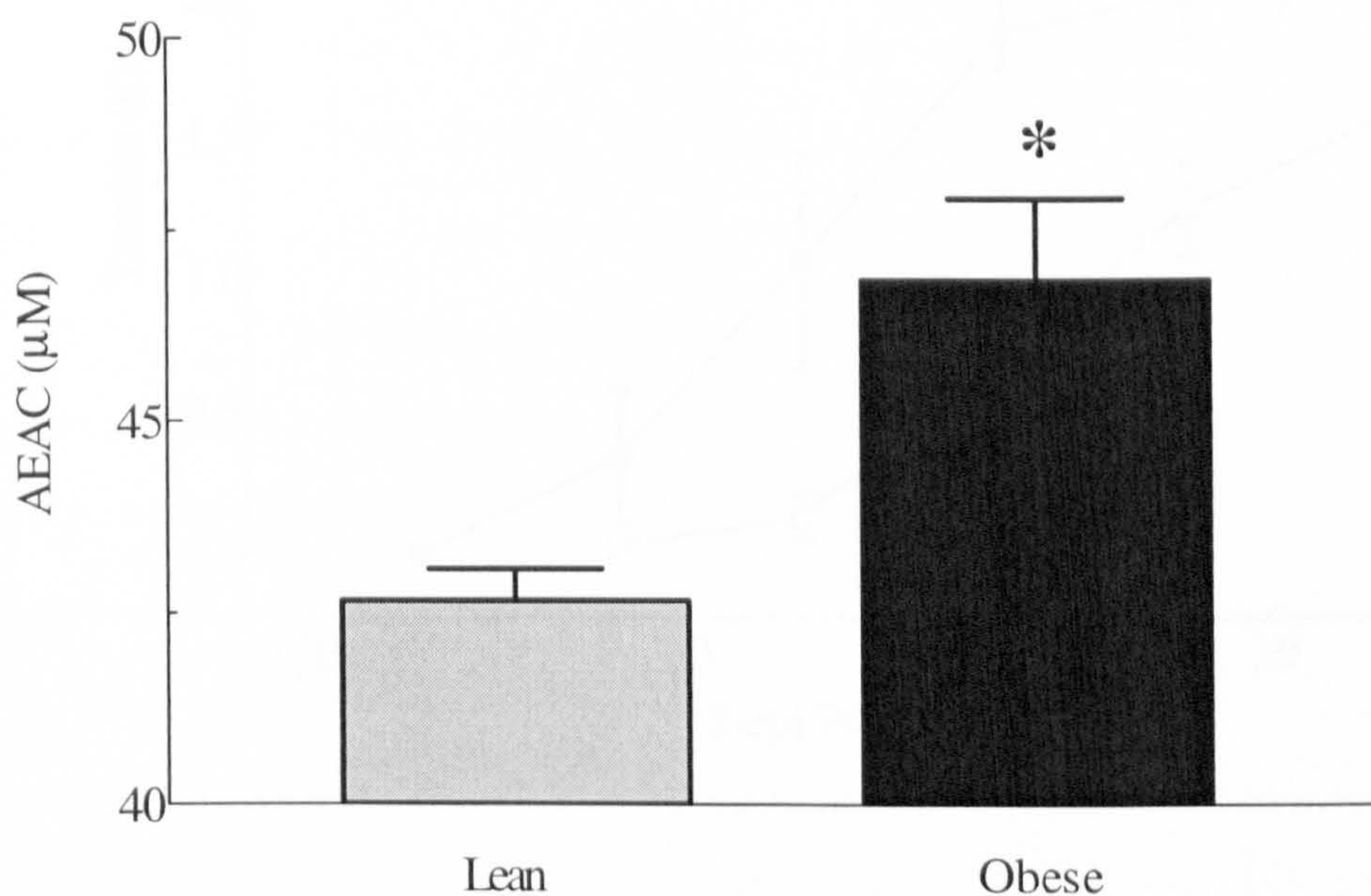


Figure 4.8 Plasma total antioxidant status (TAOS) expressed as ascorbate equivalent antioxidant concentration (AEAC) in 13 week-old lean and obese Zucker rats. Data are expressed as mean±s.e.mean of 5 determinations. Statistical significance (*) using an unpaired student t-test was considered established at $P<0.05$.

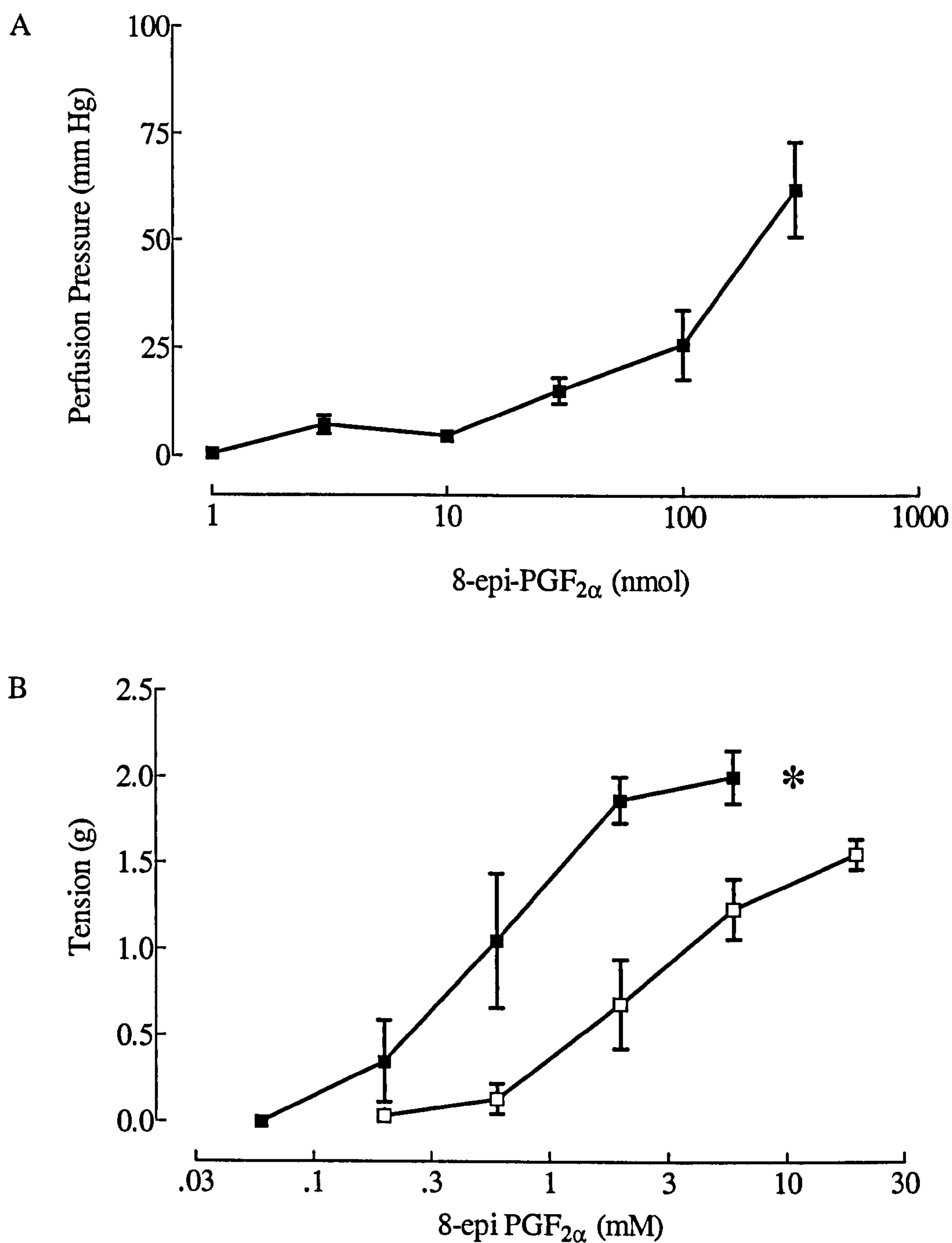


Figure 4.9 Biological effects to 8-epi prostaglandin F_{2α} (8-epi-PGF_{2α}) in Wistar rats show respectively contraction and vasoconstriction in: A. L-NAME (2.1 μmols equivalent to 300 mM) pre-treated perfused hindquarters *in situ*. B. Control (□) and L-NAME (■) treated aortic rings. Data are expressed as mean±s.e.mean of 3-4 determinations. Statistical significance (*) using two way ANOVA was considered established at $P<0.05$.

4.3 DISCUSSION

Fasting plasma insulin and glucose levels were significantly higher in the obese than in the lean littermate. Additionally, after glucose administration (during the glucose tolerance test), plasma insulin and glucose were significantly increased in the obese compared to the lean Zucker rats, and remained higher in the obese for the duration of the time course. This data compares to previous studies in obese rats, which, in contrast to lean rats, exhibited fasting hyperinsulinaemia and hyperglycaemia in addition to insulin resistance and a poor glucose disposal (Ionescu *et al.*, 1985). Insulin resistance in the obese Zucker rats has been shown in liver and adipose tissue (Terrettaz & Jeanrenaud, 1983), and, most interestingly, in skeletal muscle (Crettaz *et al.*, 1980). These results will be of particular importance in understanding the effect of insulin in the rat hindquarters (Chapter 5).

In the perfused hindquarters of the Zucker rat, endothelium-dependent, but not endothelium-independent vasodilation was enhanced (3-fold) in the obese relative to its lean littermate. This suggests that enhanced vasodilation to ACh did not simply reflect a general increase in vascular smooth muscle vasodilator reactivity in obese animals, but instead represents a selective enhancement in endothelial vasodilator function. This was the first demonstration of enhanced endothelial function in obese Zucker rats *in situ*, and supports earlier *in vitro* studies (Auguet *et al.*, 1989; Cox & Kikta, 1992; Turner *et al.*, 1995; Growcott *et al.*, 1995; Sexl *et al.*, 1995; Turner & White, 1996; Kaw *et al.*, 1998). The precise role of endothelium-derived relaxing factors remains to be established. It has been suggested that enhanced transmembrane Ca^{2+} influx might trigger NO synthesis in the obese rats (Sexl *et al.*, 1995), after experiments in rat aortic rings showed enhanced relaxation to ACh and the calcium ionophore-A23187 in obese compared to lean Zucker rats – an effect that was inhibited by L-NAME. In other studies, enhanced responses to the vasoconstrictor noradrenaline in Zucker rat aortic rings were reported to be due to abnormal

functioning of voltage sensitive Ca^{2+} channels in vascular smooth muscle (Ouchi *et al.*, 1996). If the channels are activated constantly, increased intracellular Ca^{2+} may stimulate NOS. However, in the hindquarters perfusion system, L-NAME (2.1 μmol) significantly impaired maximal vasodilation in both lean and obese Zucker rats (though the obese retained a more enhanced vasodilation to ACh in the presence of L-NAME compared to the lean). This may suggest an EDHF or prostaglandin involvement. Recent studies by our group have shown a non-NO, non-prostanoid component of endothelial vasorelaxation in obese Zucker rat aortic rings (Kaw *et al.*, 1999) that was blocked by the depolarising agent potassium chloride (increased extracellular K^+ can inhibit EDHF-induced vasorelaxation; Garland *et al.*, 1995). Other studies in the cremasteric arterioles have shown a small decrease in ACh-induced vasorelaxation in the presence of indomethacin (Frisbee & Stepp, 2001), but this still does not account for the large vasorelaxation not inhibited by a combination of L-NAME and indomethacin. EDHF is the most likely candidate for this effect, as studies in streptozotocin-induced diabetic rats have shown endothelial responses dependent upon EDHF were impaired in femoral arteries and mesentery respectively (Wigg *et al.*, 2001; Makino *et al.*, 2000). In addition, it is now generally accepted that EDHF has a greater role in the smaller vessels compared to NO; the hindquarters is a highly vascularised tissue bed populated with many small resistance vessels.

Comparison of endothelial function in the perfused hindquarters with *in vivo* measurements in 13 week-old Zucker rats anaesthetised rats showed the obese to have higher blood pressure than the lean (Ambrozy *et al.*, 1991; Pamidimukkala & Jandhyala, 1996; Laight *et al.*, 1998). It would be expected that blood pressure would be lower in the obese relative to the lean Zucker rat as endothelium-dependent vasodilation was enhanced *in situ*. This paradoxical endothelial hyperactivity and high blood pressure suggests the influence of blood borne factor(s) or autonomic controls may be responsible for hypertension in obese Zucker rats.

Blood borne factors such as endothelin have potent vasoconstrictor activity *in vivo* (see review, Haynes & Webb, 1998). The urinary excretion of endothelin and pre-proendothelin mRNA was shown to be increased in obese compared to lean Zucker rats (Turner *et al.*, 1997). Functionally, insulin has been shown to exaggerate endothelin-1-evoked Ca^{2+} responses (Hopfner *et al.*, 1998). This is an important finding, as the obese Zucker rat is hyperinsulinemic. Evidence for higher sympathetic tone in the obese Zucker rat has also been reported by Pamidimukkala & Jandhyala, (1996). Their studies showed comparable blood pressures in conscious rats, but when anaesthetised the obese rats had higher blood pressures than their lean littermates. Autonomic ganglionic blockade reduced the obese rat blood pressure to a level comparable to that of the lean Zucker rat (Pamidimukkala & Jandhyala, 1996). Therefore autonomic activation probably contributes to the hypertension observed in the obese Zucker rat. The baroreflex system responds to acute hypotension by increasing sympathetic tone to resistance as well as capacitance vessels, which increases blood pressure and venous return respectively. Elevated sympathetic activity has been shown to contribute to hypertension in hyperglycaemic obese Zucker rats in telemetry studies (Carlson *et al.*, 2000). However, the obese Zucker rat has been shown to have decreased peripheral catecholamine levels too (Levin *et al.*, 1981; Pacak *et al.*, 1995). It is thought that these differences are attributed to organ specific changes in sympathetic activity (Pacak *et al.*, 1995).

The blood volume studies confirmed that the obese Zucker rat had a significantly decreased blood volume compared to the lean Zucker rat, as previously reported (Schirardin *et al.*, 1979). Reduced blood volume has in some studies been implicated in hypertension (Gresson *et al.*, 1973; Bianchi *et al.*, 1981), where it was suggested that vasoconstriction would reduce the blood volume and that the increased muscle vascularisation in the lean could allow higher blood volumes. Additionally, a greater correlation between weight and blood volume was seen in the obese compared to the lean Zucker rat – a scenario

previously reported in genetically hypertensive rats (Huff & Feller, 1955). Reduced blood flow and blood volume has been reported in the adipose tissue of the obese Zucker rat, suggesting obese tissues are poorly perfused compared to that of the lean. If this is the case, any blood borne factors that constrict or deplete vasodilating factors may be even more damaging in the obese rat.

The idea that a key mechanism of endothelial dysfunction is NO inactivation by free radicals may be supported by the higher plasma nitrite/nitrate levels in obese rats. This supports also data where raised levels of nitrite/nitrate were found in type 2 diabetic patients (Catalano *et al.*, 1997). If there is a greater agonist-induced NO response (as observed in the perfused hindquarters) in the obese Zucker, this NO may be quickly inactivated *in vivo* but then appear as a metabolic end product in the plasma as nitrate/nitrite. Studies have shown that the glomerular filtration rate measured using inulin clearance was normal in 12-14 week rats (Kasiske *et al.*, 1985). However, as the obese Zucker rat is hyperphagic and nitrate is also a dietary metabolic end-product, caution may be needed with these results, even though the Zucker rats were fasted prior to blood collection. Furthermore, it may reflect synthesis of NO by the neuronal and inducible forms of NOS.

Higher 8-epi-PGF_{2α} levels in the obese compared to the lean Zucker rat confirmed the greater level of oxidant stress in this animal. Along with higher plasma TAOS levels in obese Zucker rats, this suggests a higher antioxidant status is perhaps already primed due to increased oxidant stress. Although it appears paradoxical that the obese Zucker rat has endothelial hyperreactivity with concomitant oxidant stress (Halliwell, 1993; Giugliano *et al.*, 1995; Harrison & O'Hara, 1995), studies have previously reported a mechanism for a ROS-mediated enhancement in agonist-stimulated endothelial function involving altered Ca²⁺ signalling (Graier *et al.*, 1996, 1997). This has been suggested to account for the

anomaly of endothelial hyperreactivity associated with early type 1 diabetes (Wascher *et al.*, 1994; Graier *et al.*, 1996). It is therefore possible that a similar scenario in the obese Zucker rat may account, at least in part, for endothelial hyperreactivity in this animal.

It is also worth considering that 8-epi-PGF_{2α} was measured in circulating plasma *in vivo* and not in the endothelial cells of the hindquarters that were perfused *in situ*. This suggests high levels of blood borne oxidant stress markers were present in the obese Zucker rat. Such increased oxidant stress due to O₂^{•-} and other ROS could bind and cause NO inactivation. This is consistent with the hypothesis that a key mechanism of endothelial dysfunction is NO inactivation by free radicals (Diederich *et al.*, 1994; Pieper *et al.*, 1996). In the young (13 weeks-old) obese Zucker rat, high ROS generation may be compensated for by increased NO synthesis following agonist-stimulation. In the older (52 weeks-old) Zucker rat, chronic oxidant stress may then contribute to the impaired endothelium-dependent vasodilation observed in other studies (Halliwell, 1993; Giugliano *et al.*, 1995; Harrison & O'Hara, 1995). It is conceivable that increased plasma levels of 8-epi-PGF_{2α} in humans with type 2 diabetes (Gopaul *et al.*, 1995) may be present before vascular complications. Indeed other markers of oxidative stress, TBARS and copper/zinc SOD have also been shown to be increased in the obese Zucker rat where hyperglycaemia, hypertension and inflammation have been absent (Poirier *et al.*, 2000).

F₂-isoprostane-containing phospholipids show distortion of the fluidity and integrity of plasma membranes, and may therefore interfere with normal physiological processes (Morrow *et al.*, 1992). These findings are consistent with endothelial dysfunction involving the alteration of transmembrane signalling mechanisms and Ca²⁺ homeostatic mechanisms that cause an increase in the release of paracrine factors (Schilling & Elliott, 1992). The physiological role of 8-epi-PGF_{2α} and related prostanoids is unknown. However, my studies confirmed a vasoconstriction by 8-epi-PGF_{2α} in aortic rings and the hindquarters

(Fukunaga *et al.*, 1993; Banerjee *et al.*, 1992; Kang *et al.*, 1993). L-NAME treatment enhanced vasoconstriction, suggesting that NO inactivation by ROS, and the direct vasoconstriction by 8-epi-PGF_{2α} produce additive endothelial damage.

The data in this chapter show the 13 week-old obese Zucker rat with a hyper-endothelial response, probably due to an increased NO action suggested by higher plasma nitrate/nitrite levels. This may be a compensation response to the increased sympathetic drive and oxidant stress present in the obese Zucker rat that are realised by raised 8-epi-PGF_{2α} and TAOS measurements. The increased redox stress may lead to increased vasoconstriction *in vivo*, indirectly by the inactivation NO and directly by 8-epi-PGF_{2α}-induced constriction. The raised plasma insulin and glucose levels and a poor glucose disposal observed in the obese Zucker rats may be also be indicative of poor NO responses *in vivo*. Indeed, impaired glucose disposal rates have been shown in eNOS knockout mice (Shankar *et al.*, 2000). If in these studies increased ROS causes inactivation of NO, then glucose disposal may be impaired, resulting in higher insulin and eventually higher glucose levels. Conversely, hyperglycaemia in addition to increased NO and ROS levels may also lead to disruption of cellular signalling and in turn endothelial function. The relationship between insulin and NO in the Zucker rat however remains inconclusive. Studies on cultured vascular endothelial cells isolated from 12 week-old Zucker rats have demonstrated that insulin increased levels of eNOS mRNA and protein, and increased eNOS activity in lean but not in insulin-resistant obese littermates (Kuboki *et al.*, 2000). It follows that insulin is an unlikely to be involved in the compensation mechanism that induces increased NO in the obese rats.

Chapter 5

*Effect of insulin on endothelium-
dependent vasodilation in the perfused
hindquarters and aortic rings of the obese
Zucker rat*

5.1 INTRODUCTION

Initial findings that suggested insulin was involved in vasodilation appeared in patient studies where insulin administration caused marked hypotension associated with hypoglycaemia (Miles & Hayter, 1968). More recently studies have shown that insulin can induce hypotension in subjects where glucose levels were kept constant. One of the first studies to support the vasodilator function of insulin was by Liang (1982), who showed it decreased vascular resistance and increased muscle blood flow. Further studies in normotensive humans have demonstrated an insulin sensitive redistribution of blood flow to more insulin sensitive sites (Baron *et al.*, 1993). The mechanism of insulin-mediated vasodilation remains unclear, but is likely to be via insulin's modulation of NO-dependent vasodilation (Steinberg *et al.*, 1994). There is also evidence of a positive relationship between basal vascular endothelial NO production and insulin sensitivity in healthy human volunteers (Petrie *et al.*, 1996). Insulin stimulated production of NO is through activation of phosphatidylinositol 3-kinase (PI 3-kinase) (Zeng & Quon, 1996), via IRS proteins in the insulin signalling pathway. However, insulin also stimulates cGMP production directly (Kahn *et al.*, 1998), and can augment NO-induced cGMP production in vascular smooth muscle cells (Kahn *et al.*, 2000).

There is paradoxical evidence of insulin-induced vasoconstriction from studies in sympathectomised rats (Edwards & Tipton, 1989). Here, insulin increased blood pressure in control, but not in sympathectomised or hexamethonium treated rats, suggesting insulin-induced vasoconstriction is modulated by the sympathetic nervous system. Indirect vasoconstrictor function of insulin has been shown by its ability to desensitise vascular responsiveness to noradrenaline at β -adrenoceptors. Studies reported that the β -adrenoceptor G proteins may be phosphorylated in response to insulin, leading to reduced signalling (Hadcock *et al.*, 1992).

In later studies, insulin potentiated a noradrenaline-induced tone in the rabbit facial artery by activation of protein kinase C and tyrosine kinase (Henrion & Laher, 1994). This suggests insulin can also tilt the vascular balance back toward greater vasoconstriction when sympathetic mediators are raised. More chronic studies of insulin's action have shown increased gene expression (Oliver *et al.*, 1991) and release (Hattori *et al.*, 1991) of the potent vasoconstrictor endothelin-1 from endothelial cells.

In diabetic disease, the notion that insulin induces vasodilation and that this is impaired in diabetics is most possible. As a vasodilator effect of insulin is potentially a critical factor to its metabolic function, i.e. the efficient disposal of glucose at insulin sensitive sites. It can also be hypothesised that defective insulin-induced vasodilation due to insulin resistance will further impede insulin's metabolic function. Certainly the NO-dependent enhancement of endothelium-dependent vasodilation by insulin, which has been observed experimentally *in vivo* (Steinberg *et al.*, 1994; Scherrer *et al.*, 1994) may be abnormal in diabetes (Steinberg *et al.*, 1996). It should be noted, however, that, increased sympathetic tone inducing VSM relaxation via α -adrenoceptor stimulation is detrimental to glucose disposal in resistance and small conductance arteries of the skeletal muscle. Chronic sympathetic stimulation, such as that observed in the Zucker rat (Pamidimukkala & Jandhyala, 1996), can cause desensitisation of the adrenoceptor and in turn alter glucose disposal.

The aim of these studies was, firstly to investigate the action of insulin in a macrovascular and microvascular preparation as NO-induced vasodilator responses in diabetics are known to be impaired in both conduit and resistance vessels (Diederich *et al.*, 1994). Secondly, an attempt was made to understand if insulin resistance or the impaired glucose tolerance seen in the obese Zucker contributed to endothelial dysfunction and whether this could then in turn be linked to disease progression from reduced glucose disposal to hyperglycaemia and consequently diabetes.

5.2 RESULTS

5.2.1 Initial validation studies investigating concentration and incubation times of insulin in Wistar rat aortic rings.

Incubation with insulin (100 nM) for 20 min increased the endothelium-dependent vasorelaxation to ACh compared to BSA treated paired rings (Fig. 5.1A; Table 5.1). A 10 min treatment with insulin (100 nM) had no significant effect, while a 30 min treatment was determined to be no more effective than a 20 min treatment. Therefore 20 min was chosen for subsequent studies. With respect to pre-treatment, 20 min with insulin (10 nM) was without effect ($P>0.05$) (Fig. 5.1B; table 5.2), whereas a 20 min with insulin (100 nM and 1000 nM) caused a similar increase in endothelium-dependent vasodrelaxation – therefore 100 nM was adopted in subsequent studies. In contrast to ACh, endothelium-independent vasorelaxation to SNP ($n=6$) vehicle control ($pD_2 = 7.60 \pm 0.12$, $n=6$) was not affected by insulin (100 nM) ($pD_2 7.78 \pm 0.08$, $n=6$; $P>0.05$).

	Insulin (nM)			
	0	10	100	1000
pD_2	7.16 ± 0.13	7.09 ± 0.22	7.13 ± 0.12	7.14 ± 0.11
$E_{max} (\%)@ 1 \mu M$	70.0 ± 5.4	67.7 ± 8.7	78.6 ± 5.9	84.9 ± 2.9

Table 5.1 Vasorelaxation to ACh (0.0001-10 μM) in Phe pre-contracted Wistar rat aortic rings following treatment with insulin (10, 100 & 1000 nM) or its vehicle (BSA 0.1 %) for 20 min. Data are expressed as mean \pm s.e.mean of 4-5 determinations.

5.2.2. Effects of insulin on the contractile reactivity in Zucker rat aortic rings

Insulin (100 nM) did not significantly change the Phe elicited contractions from that of the BSA vehicle responses, either in the presence or absence of L-NAME, in lean and obese Zucker rats (Fig. 5.2; table 5.3). Phe-induced contractions were similar in lean and obese rings, whether BSA or insulin treated ($P>0.05$). However, treatment with L-NAME induced

slight leftward shift in both insulin and BSA treated rings from obese and lean rats respectively ($P<0.05$), and only slightly increased the E_{\max} values from obese Zucker rats (Fig. 5.2; table 5.3).

	Insulin		Control	
Time (min)	pD_2	E_{\max} (%)	pD_2	E_{\max} (%)
10	7.28±0.11	81.9±4.3	7.17±0.11	83.2±2.9
20	7.51±0.11	86.4±4.1	7.31±0.12	79.2±3.4
30	7.44±0.10	92.8±1.2	7.30±0.11	85.6±2.3

Table 5.2 Timecourse for insulin (100 nM) or its vehicle (BSA 0.1%) in Phe pre-contracted Wistar rat aortic rings effects on vasorelaxation to ACh (0.0001-10 μ M). Data are expressed as mean±s.e.mean of 6-8 determinations. E_{\max} was reached at 1 μ M.

	Obese		Lean	
Treatment	pD_2	E_{\max} (%)	pD_2	E_{\max} (%)
BSA	7.09±0.1	2.3±0.07	7.38±0.12	2.3±0.09
Insulin	7.23±0.08	2.3±0.06	7.32±0.13	2.0±0.08
BSA + NAME	7.99±0.14* Ψ	2.4±0.07	7.47±0.15	2.3±0.01
Insulin + NAME	8.11±0.11* Ψ	2.6±0.07	7.64±0.11	2.3±0.08

Table 5.3 Vasoconstriction to Phe (0.01-10 μ M) following 20 min treatment with insulin (100 nM) or its vehicle (BSA 0.1%) in the presence or absence of L-NAME (300 μ M) in obese and lean Zucker rat aortic rings. Data are expressed as mean±s.e.mean of 4-5 determinations. E_{\max} was reached at 3 μ M. * $P<0.05$ with respect to corresponding treatment in the lean group; $^{\dagger}P<0.05$ with respect to corresponding BSA group; $^{\Psi}P<0.05$ with respect to corresponding treatment in the absence of L-NAME.

5.2.3 Effects of insulin on the vasodilator reactivity in Zucker rat aortic rings

ACh-induced vasorelaxation was slightly greater in the lean compared to the obese Zucker rats ($P>0.05$). Insulin enhanced this relaxation in the obese but not the lean group (Fig. 5.3;

table 5.4). This resulted in a greater ACh-induced vasorelaxation in obese compared to lean rings where insulin was present ($P<0.05$). SNP-induced vasorelaxation was unaffected by treatment with insulin in both lean and obese ($P>0.05$). However, pD_2 values were different between lean and obese treated with insulin ($P<0.05$) (table 5.5).

	Obese		Lean	
Treatment	pD_2	E_{\max}	pD_2	E_{\max}
BSA	7.05 ± 0.05	85.3 ± 2.1	7.24 ± 0.06	89.6 ± 1.5
Insulin	$7.37\pm0.11^{*\dagger}$	92.3 ± 2.8	7.15 ± 0.06	90.0 ± 2.2

Table 5.4 Vasorelaxation to ACh (0.0001-10 μ M) in Phe pre-contracted obese and lean Zucker rat aortic rings following 20 min treatment with insulin (100 nM) or its vehicle (BSA 0.1 %). Data are expressed as mean \pm s.e.mean of 5-7 determinations. E_{\max} was reached at 1 μ M. * $P<0.05$ with respect to corresponding treatment in the lean group; $\dagger P<0.05$ with respect to corresponding BSA group.

	Obese		Lean	
Treatment	pD_2	E_{\max}	pD_2	E_{\max}
BSA	7.23 ± 0.06	94.7 ± 1.2	7.24 ± 0.06	96.8 ± 1.2
Insulin	$7.22\pm0.05^*$	93.6 ± 1.4	7.45 ± 0.08	95.9 ± 1.6

Table 5.5 Vasorelaxation to SNP (0.01-100 μ M) in Phe pre-contracted obese and lean Zucker rat aortic rings following 20 min treatment with insulin (100nM) or its vehicle (BSA 0.1 %). Data are expressed as mean \pm s.e.mean of 4-6 determinations. E_{\max} was reached at 3 μ M. * $P<0.05$ with respect to corresponding treatment in the lean group.

5.2.4 Effects of insulin on contractile reactivity in Zucker rat perfused hindquarters

Insulin had no effect on basal perfusion pressure (PP) in obese (BSA: 70.8 ± 6.7 , $n=3$; insulin: 60.5 ± 1.4 mm Hg; $n=3$, $P>0.05$) or lean rats (BSA: 65.6 ± 3.7 mm Hg, $n=4$; insulin: 87.1 ± 13.8 mm Hg; $n=4$, $P>0.05$). Similarly, insulin had no effect on L-NAME-elevated PP

in obese (BSA: 61.2 ± 3.5 mm Hg, $n=4$; insulin: 59.5 ± 4.4 mm Hg; $n=3$, $P>0.05$) or lean rats (BSA: 95.9 ± 30.6 mm Hg, $n=3$; insulin: 69.9 ± 2.2 mm Hg; $n=3$, $P>0.05$).

Phe-induced comparable constrictions in insulin (100 nM) and BSA treated hindquarters in the obese, but not lean, Zucker rats. The pD_2 values showed a significant decrease in Phe-induced constrictions by insulin. Changes in E_{max} , however, were unaffected (Fig. 5.4; table 5.6). In the presence of L-NAME, insulin provoked an enhanced Phe-induced constriction in the obese ($P<0.05$), but depressed the responses in lean preparations ($P>0.05$) (Fig. 5.4; table 5.6). Insulin and BSA treated preparations showed a leftward shift in Phe-induced constrictions when treated with L-NAME in both lean and obese hindquarters.

	Obese		Lean	
Treatment	pD_2	E_{max}	pD_2	E_{max}
BSA	7.20 ± 0.02	197.3 ± 4.4	7.21 ± 0.02	208.6 ± 6.5
Insulin	7.15 ± 0.08	215.8 ± 7.5	$7.02 \pm 0.02^\dagger$	217.3 ± 4.8
BSA + NAME	$7.89 \pm 0.01^{*\Psi}$	200.1 ± 7.2	$8.25 \pm 0.02^\Psi$	209.2 ± 9.7
Insulin + NAME	$8.20 \pm 0.16^{\dagger\Psi}$	217.7 ± 8.4	$8.13 \pm 0.02^{\dagger\Psi}$	$193.5 \pm 4.3^\Psi$

Table 5.6 Vasoconstriction to Phe (0.01-100 nmol) infusions in obese and lean Zucker rat perfused hindquarters *in situ* following 20 min treatment with insulin (100 nM) or its vehicle (BSA 0.1%) in the presence or absence of L-NAME (300 μ M). E_{max} was reached at 100 nmol in L-NAME treated and at 3 μ mol in untreated preparations. Data are expressed as mean \pm s.e. mean of 3-4 determinations. * $P<0.05$ with respect to corresponding treatment in the lean group; $^\dagger P<0.05$ with respect to corresponding BSA group; $^\Psi P<0.05$ with respect to corresponding treatment in the absence of L-NAME.

5.2.5 Effects of insulin on vasodilator reactivity of the Zucker rat perfused hindquarters

Insulin had no effect on basal perfusion pressure (BPP) in obese or lean animals ($P>0.05$), or on the Phe infusion-elevated PP ($P>0.05$) (table 5.7).

	Basal PP		Phe elevated PP	
Treatment	vehicle	Insulin	vehicle	Insulin
Obese BPP (mm Hg)	30.3±2.7	27.5±3.2	129±19.8	157.6±14.7
Lean BPP	33.3±1.5	31.4±3.6	160.0±11.6	163.7±19.2

Table 5.7 Basal and Phe elevated perfusion pressures in lean and obese Zucker rats following insulin (100 nM) or its vehicle (BSA 0.1 %) infusion. Data are expressed as mean±s.e.mean of 9-12 determinations.

Vasodilation to ACh was impaired by insulin in obese and lean animals ($P<0.05$) (Fig. 5.5; table 5.8). A rightward shift of the ACh curve and blunted E_{max} was seen in the obese rats, and a smaller rightward shift was seen in the lean rats ($P<0.05$). In contrast, obese and lean vasodilation to SNP was unaffected by insulin ($P>0.05$) when compared by two way ANOVA (table 5.9). However pD_2 values show vasodilation to SNP was impaired by insulin in the obese and enhanced in the lean animals ($P<0.05$) (table 5.9).

	Obese		Lean	
Treatment	pD_2	E_{max}	pD_2	E_{max}
BSA	10.65±0.02*	49.7±3.1	10.84±0.03	41.3±2.3
Insulin	10.18±0.04*†	44.3±3.9	10.43±0.03†	44.8±2.8

Table 5.8 Vasodilation to bolus dose ACh (0.0001-10 nmol) in Phe pre-constricted obese and lean Zucker rat perfused hindquarters *in situ* following 20 min treatment with insulin (100 nM) or its vehicle (BSA 0.1%). E_{max} was reached at 10 nmol. Data are expressed as mean±s.e.mean of 5-7 determinations. * $P<0.05$ with respect to corresponding treatment in the lean group; † $P<0.05$ with respect to corresponding BSA group.

	OBESE		LEAN	
Treatment	pD_2	E_{max}	pD_2	E_{max}
BSA	$8.76 \pm 0.05^*$	42.7 ± 2.3	8.57 ± 0.01	43.5 ± 3.1
Insulin	$8.63 \pm 0.02^{*\dagger}$	44.7 ± 4.3	$8.82 \pm 0.02^\dagger$	40.0 ± 3.0

Table 5.9 Vasodilation to bolus dose SNP (0.01-100 nmol) in Phe pre-constricted obese and lean Zucker rat perfused hindquarters *in situ* following 20 min treatment with insulin (100 nM) or its vehicle (BSA 0.1%). Data are expressed as mean±s.e.mean of 4-5 determinations. E_{max} was reached at 100 nmol. $*P<0.05$ with respect to corresponding treatment in the lean group; $^\dagger P<0.05$ with respect to corresponding BSA group.

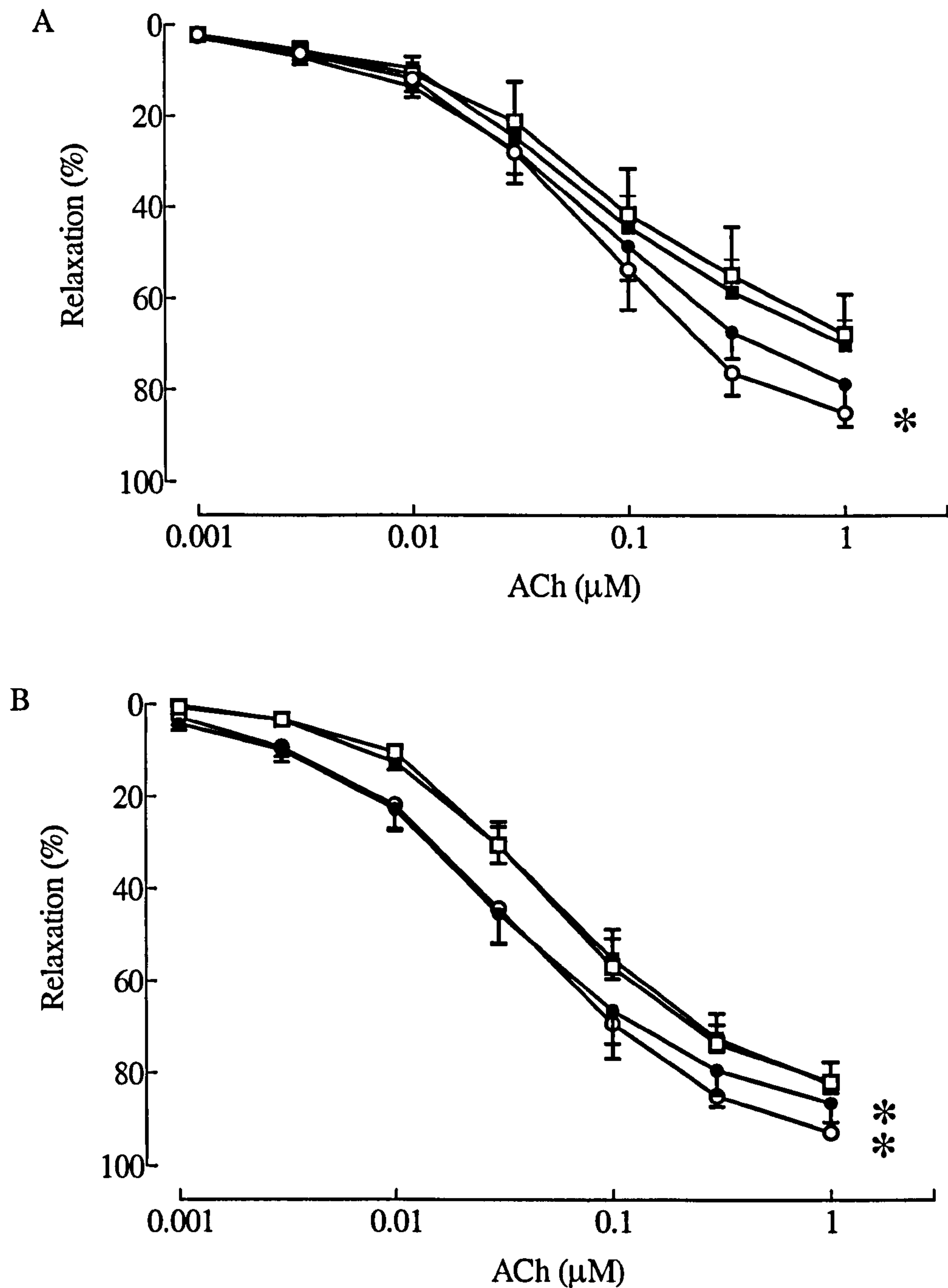


Figure. 5.1 Relaxation to acetylcholine (ACh) in the isolated Wistar rat aorta: Effect of insulin. A. Comparison of vehicle (\blacksquare) and insulin concentrations 10 (\square), 100 (\bullet) and 1000 (\circ) nM. B. Comparison of mean vehicles (\blacksquare) and insulin (100 nM) incubation times 10 (\square), 20 (\bullet), and 30 (\circ) min. Data are expressed as mean \pm s.e.mean of 5-8 determinations. Statistical significance (*) using two way ANOVA was considered established at $P<0.05$.

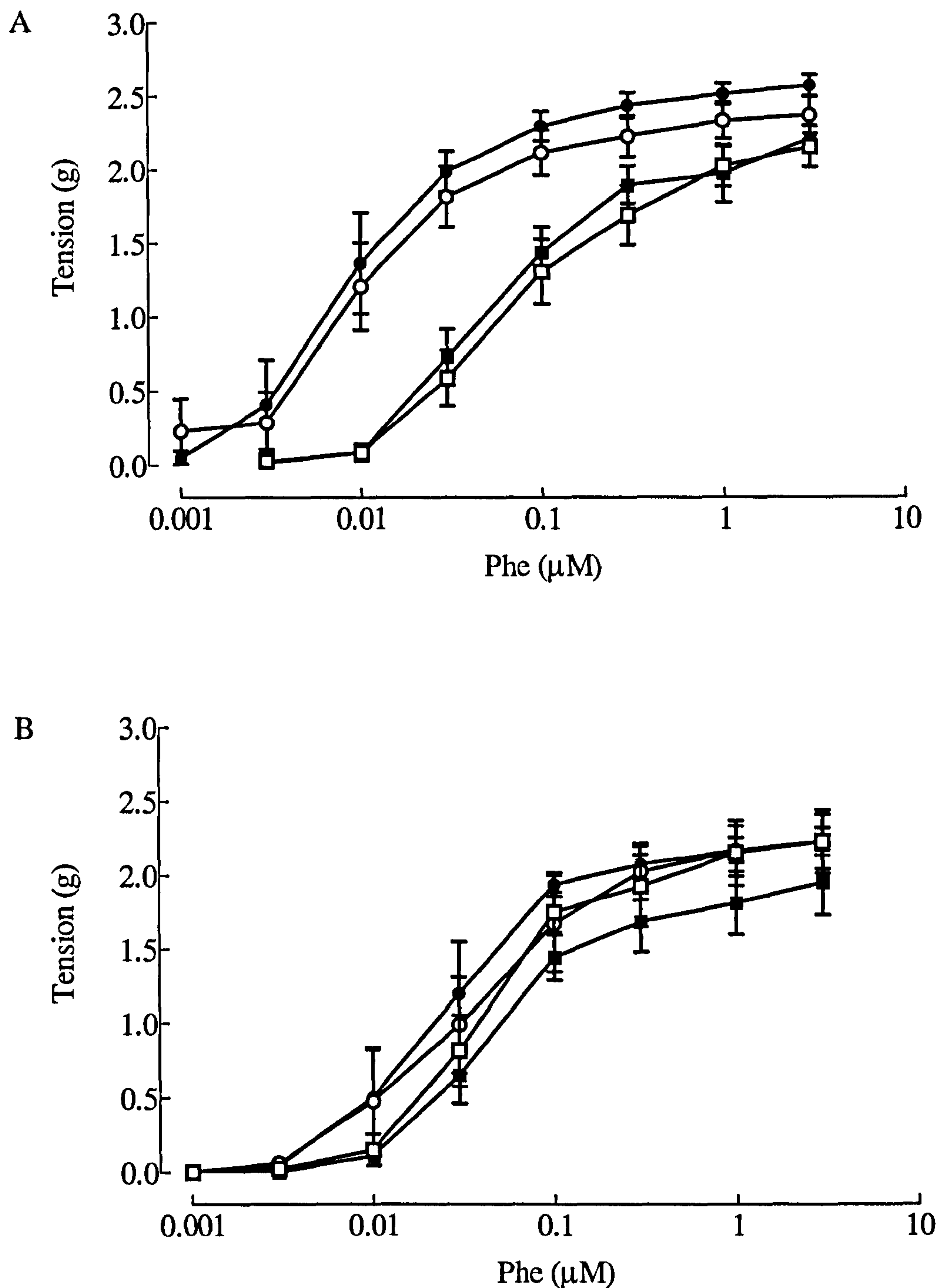


Figure 5.2 Effects of insulin (100 nM) and its vehicle BSA (0.1 %) on phenylephrine (Phe, 0.001-3 μM) cumulative concentration responses in 13 week-old lean and obese Zucker rat aortic rings. Comparison of A. obese rats B. lean rats treated with BSA (\square), and insulin (\blacksquare), BSA+L-NAME (\circ), and insulin+L-NAME (\bullet). Data are expressed as mean \pm s.e.mean of 6-7 determinations.

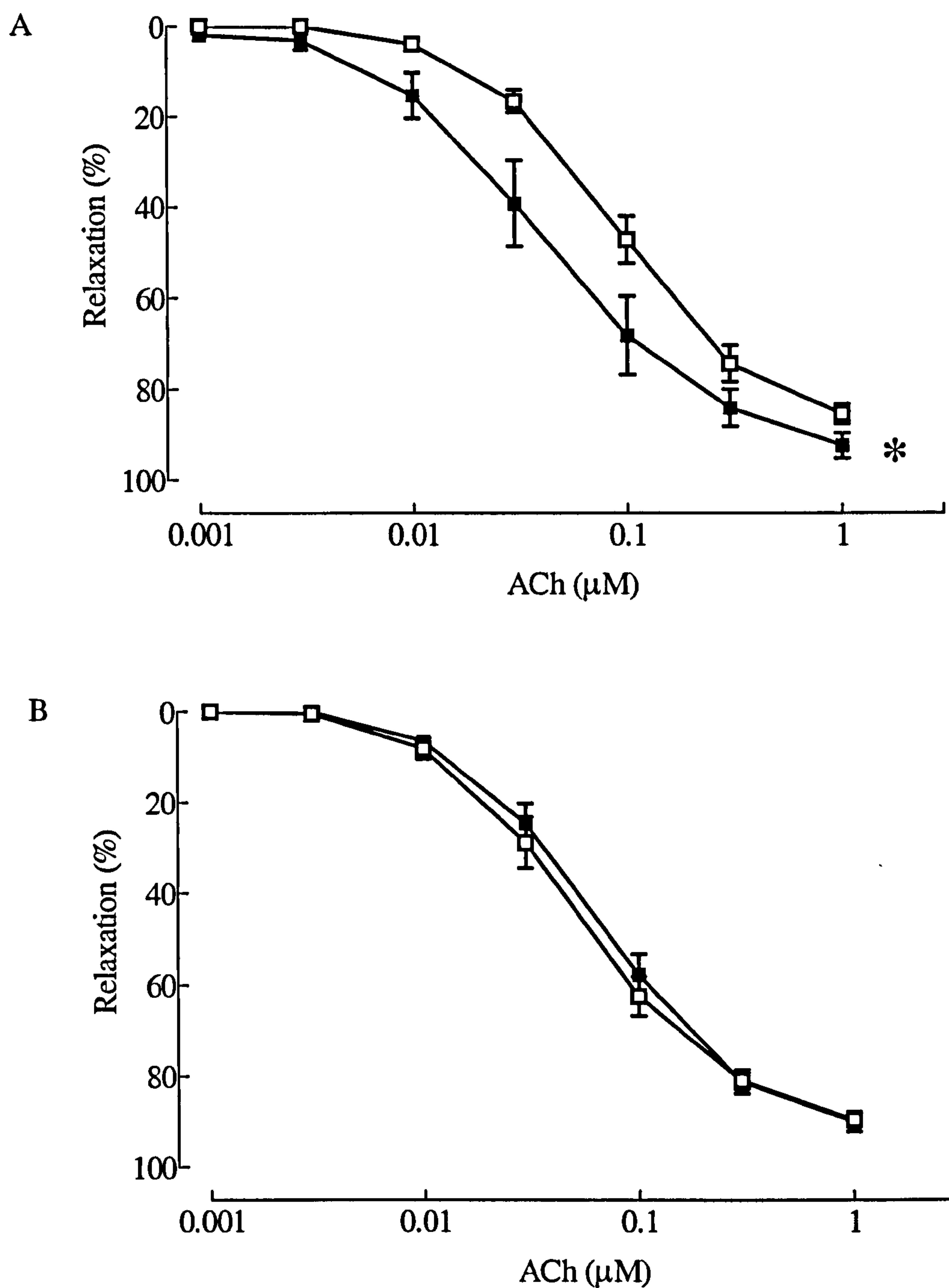


Figure 5.3 Effects of insulin (100 nM) and its vehicle BSA (0.1 %) on acetylcholine (ACh, 0.001-1 μM) cumulative concentration responses in 13 week-old lean and obese Zucker rat aortic rings. Comparison of A. obese rats or B. lean rats treated with BSA (□) and insulin (■). Data are expressed as mean±s.e.mean of 6-7 determinations. Statistical significance (*) using two way ANOVA was considered established at $P<0.05$.

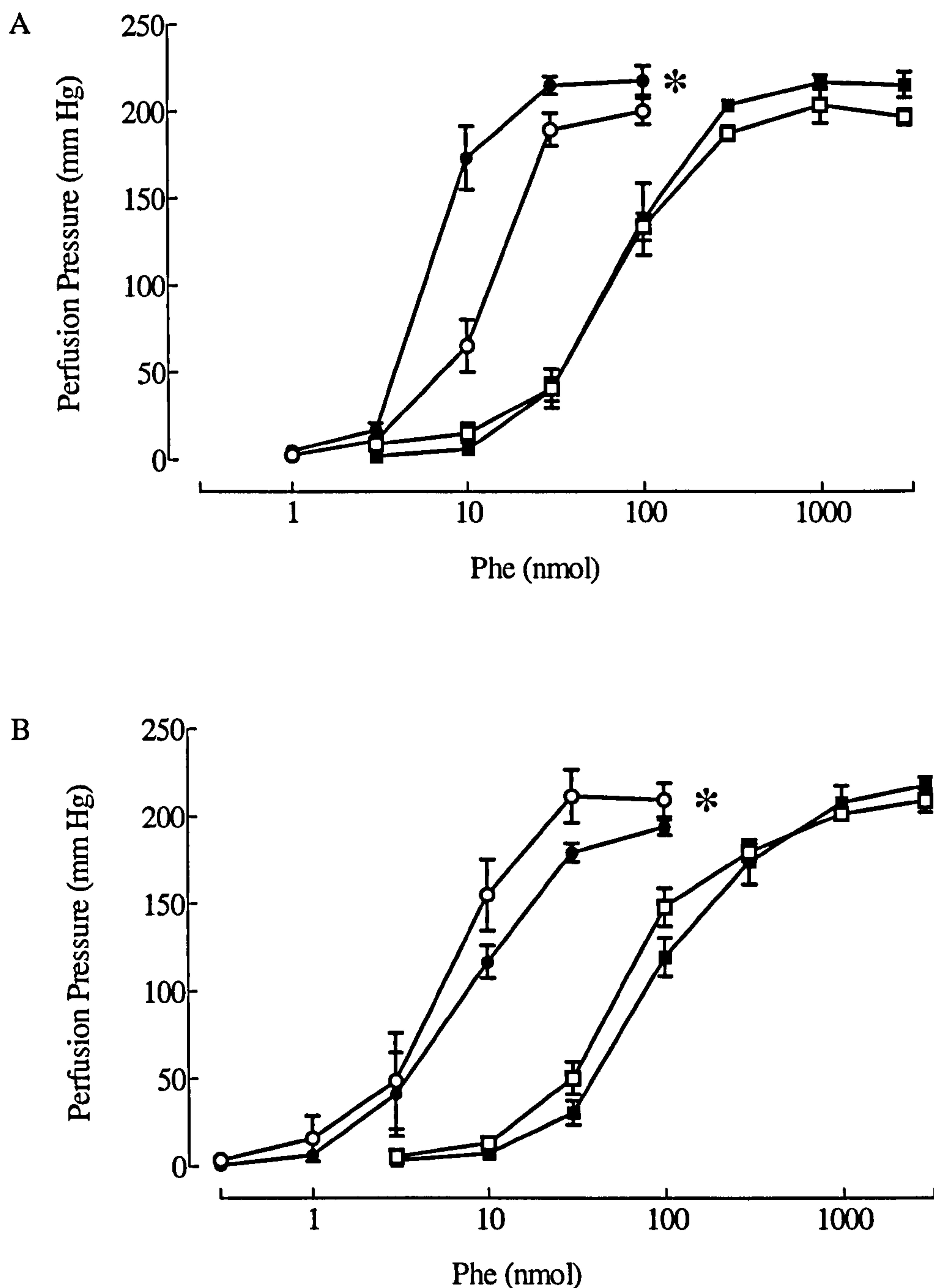


Figure 5.4 Effects of insulin (100 nM) and its vehicle BSA (0.1 %) on phenylephrine (Phe, 0.3-3000 nmol) cumulative dose responses in control and L-NAME (2.1 μ mols equivalent to 300 μ M) treated preparations in 13 week-old lean and obese Zucker rats perfused hindquarters *in situ*. Comparison of A. obese rats and B. lean rats treated with BSA (□), insulin (■), BSA+L-NAME (○) or insulin+L-NAME (●). Data are expressed as mean \pm s.e.mean of 3-4 determinations. Statistical significance (*) using two way ANOVA was considered established at $P<0.05$.

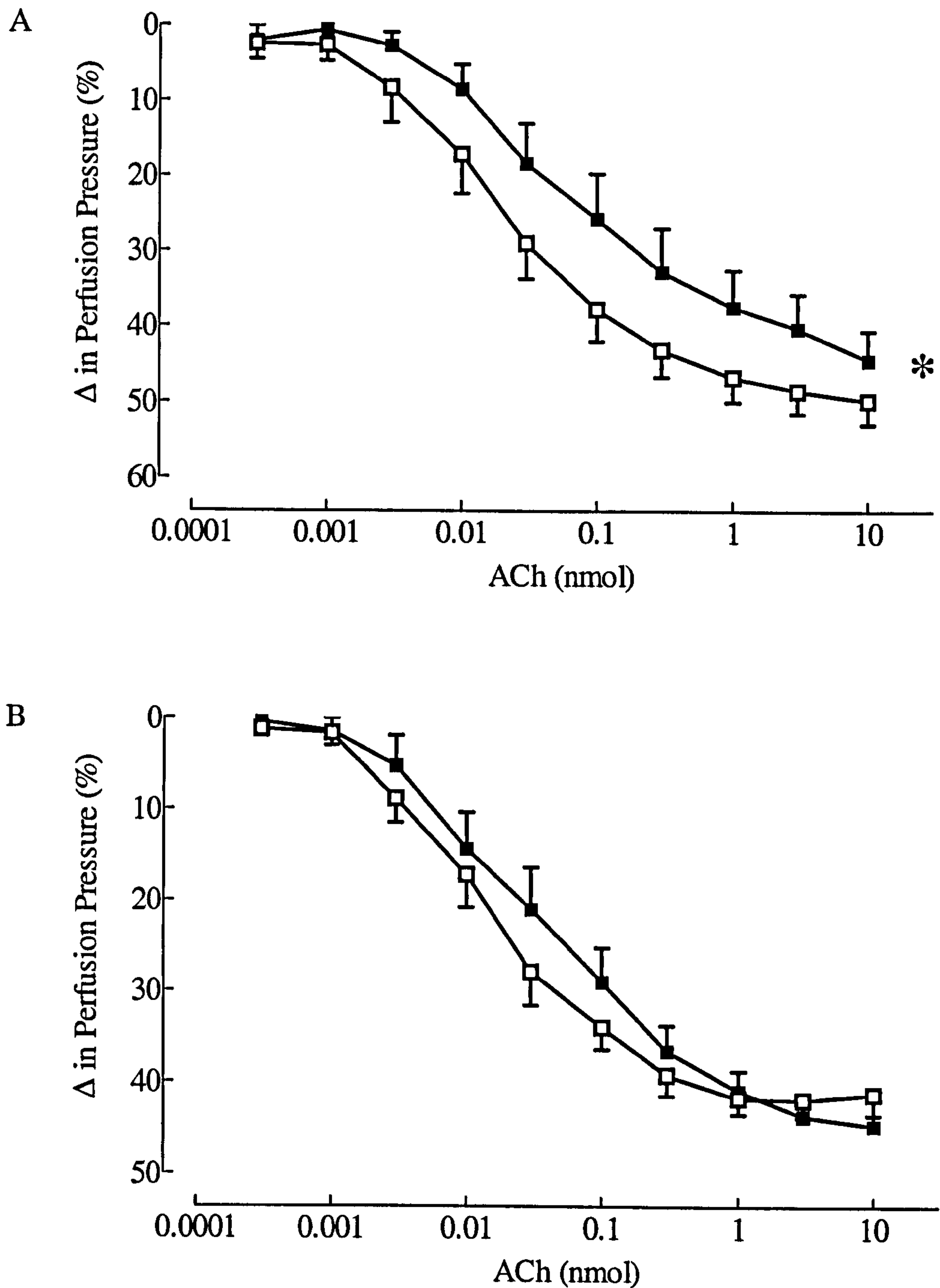


Figure 5.5 Effects of insulin (100 nM) and its vehicle BSA (0.1 %) on acetylcholine (ACh, 0.0003-10 nmol) dose responses in 13 week-old lean and obese Zucker rats perfused hindquarters *in situ*. Comparison of A. obese rats B. lean rats treated with BSA (□) and insulin (■). Data are expressed as mean±s.e.mean of 5-7 determinations. Statistical significance (*) using two way ANOVA was considered established at $P<0.05$.

5.3 DISCUSSION

Initial studies in rat aortic rings provided for the optimum insulin concentrations and timecourse for all further studies. After timed incubations (10-30 min) with insulin (100 nM), the 20 min interval was chosen for subsequent experiments. Concentration ranging (10-1000 nM) showed 100 nM to cause a maximal increase in endothelium-dependent vasorelaxation and was therefore adopted in subsequent studies. It is important to note that endothelium-independent vasorelaxation responses were not significantly affected by insulin (100 nM), indicating an endothelial-dependent action of insulin. The high pharmacological concentration of insulin required in these studies for an endothelial-dependent effect may be due to variability in tissue responsiveness to insulin outside the normal *in vivo* metabolic environment and/or heterogeneity in the density of vascular insulin receptors (Zeng & Quon, 1996; Muggeo *et al.*, 1977; Bar *et al.*, 1979). It may also be due to differences in the species specificity, as the insulin used in these experiments was recombinant human – said to be effective on all mammalian cells as there is little difference in rat and human insulin homology. However, a study has shown that insulin receptors of mammals, but not those of rodents, share the same epitope(s) (Tong *et al.*, 1994). This suggests that the human insulin used in these experiments may not have efficiently stimulated the rat insulin receptors. Alternatively, it is possible that the vascular effects of insulin may be at least in part mediated by the insulin-like growth factor receptor system, for which insulin exhibits a somewhat lower potency (Walsh *et al.*, 1996; Sowers, 1996).

In rat aorta studies, insulin had no effect on Phe elicited contractions compared to BSA, even in the presence of L-NAME, in either obese or lean Zucker rings. Treatment with L-NAME induced a leftward shift in both insulin and BSA treated rings from the obese and lean rats. However, this shift was significantly greater in the obese, perhaps suggesting there was a greater basal NO release in the obese. Additionally, insulin enhanced the ACh-

induced relaxation in the obese but not the lean group, resulting in a greater vasorelaxation in the obese compared to the lean rings where insulin was present. These findings suggest that the obese has a greater basal NO release in the aorta, which is more sensitive to insulin-induced mediation. It may be that the obese has a higher basal NO release due to the hyperinsulinaemia *in vivo*. Studies by Baron (1994) showed that increases in skeletal muscle blood flow were dependent on the exposure time to insulin. Here, insulin mediated a NO-induced vasodilation over a 4-9 hour exposure. However, although this time period may be physiologically consistent with metabolism in normal animals, when compared to the hyperphagic obese Zucker rats, this exposure is small. The obese Zucker rat may be effectively in an almost permanent post prandial state and therefore exhibit continual NO-mediated vasodilation due to constantly evoked insulin release. It could then be reasoned that the insulin mediated NO-induced vasodilation is a physiological factor in glucose disposal, a process that is particularly active in the obese rat. The increased ACh-induced vasorelaxation in the presence of insulin may then be a further upregulation of this physiological process.

Vasorelaxation to SNP in both lean and obese rats, was unaffected by insulin treatment when the aortic ring response curves were compared by 2 way ANOVA. However, the pD_2 values showed a significant difference between lean and obese response after treatment with insulin. This difference was due to a greater SNP-induced relaxation in the lean, as the obese relaxations were completely unaffected by insulin treatment. This difference may be due to a non-specific effect of insulin in the lean rats which causes the vascular smooth muscle to be more sensitive to NO-induced vasodilation, possibly via insulin's stimulation cGMP production itself (Kahn *et al.*, 1998). However, if this was the case one would expect the ACh-induced vasorelaxation to also be enhanced by insulin, and it was not. Again this would suggest less NO was released/synthesised in the vasculature of the lean, as there was not an enhanced response. Alternatively, insulin may have mediated a vasoconstriction to

compensate for this increased vascular smooth muscle sensitivity via another non-specific endothelium-independent mechanism.

In contrast to the aortic ring data, the hindquarter perfusion preparations showed there was no difference between control and insulin treated Phe-induced vasoconstriction responses in the lean and obese rats. However, in the presence of L-NAME, the obese vasoconstriction curve to Phe was shifted further leftwards in insulin treated as opposed to control preparations. This suggests that NO, when not inhibited by L-NAME, compensates for the increased constriction evoked by insulin treatment. This may be possible if insulin has a dual role, causing both vasodilation through a NO-dependent pathway and constriction through another pathway. Numerous studies have shown that insulin causes acute vasodilation through a NO-dependent pathway (Taddei *et al.*, 1995). However, insulin has also been shown to potentiate vasoconstriction to noradrenaline (Henrion & Laher, 1994) through a specific pathway involving tyrosine kinase and PKC activation in rabbit facial arteries. In addition, insulin has been shown to increase plasma noradrenaline by causing sympathetic nerve terminal spillover (Yki-Jarvinen & Utriainen, 1998). This may not be applicable in the hindquarters perfused preparation, as the CNS is severed the nerve terminals are, however, still present *in situ*.

Vasodilation to ACh was more significantly impaired by insulin in the obese compared to lean rat preparations. It may be argued that this would be expected as the vasodilation was greater in the control preparations in the obese than in the lean ones (although this is not consistently represented – pD_2 values are comparable, probably due to the increased E_{max} in the obese) and therefore any inhibition would be marked. It may also suggest further evidence of the involvement of the dual vasoconstrictor mechanism. Though, as SNP-induced vasodilation was unaffected by insulin in both obese and lean preparations, this would exclude a non-specific effect of insulin and point to insulin-evoked blunting of ACh-

stimulated NO biosynthesis/release in the hindquarters of the obese Zucker rat. This impaired insulin mediated vasodilation is consistent with findings from diabetic patient studies *in vivo* (Johnstone *et al.*, 1993). However if the pD_2 values are considered this impaired ACh vasodilation may be an endothelial-independent effect on VSM. It is interesting that if this is an endothelial-independent effect by insulin that it inhibits vasodilation in obese and enhances vasodilation in the lean. In addition, these *in situ* experiments have revealed that in the absence of insulin, there is an enhanced basal NO release in the hindquarters. Therefore insulin is not only a poor mediator of NO-induced vasodilation in diabetics subjects, but it probably abrogates basal NO-induced vasodilation as well.

In summary, the macrovascular and microvascular preparations used in these studies have revealed that basal NO release was enhanced in the obese compared to the lean rats. However, agonist-induced vasodilation was enhanced only in the obese hindquarters, which was abrogated by the addition of insulin. In contrast, the agonist-induced vasodilation was similar in lean and obese rings, but was significantly enhanced in the obese by insulin. The different responses from the macro or microvascular preparations show the importance of using insulin sensitive vascular beds for the investigation of insulin mediated vasoactive responses. More importantly, insulin has been shown to differentially regulate systemic and skeletal muscle vascular resistance (Baron & Brechtel, 1993). The skeletal bed and organs that require glucose uptake are subjected to greater blood redistribution and shunting, as their metabolic needs are more crucial than a large conduit vessel.

Chapter 6

*Modulation of endothelial function,
oxidant stress and insulin resistance by
antioxidant and pro-oxidant treatment in
the obese Zucker rat*

6.1 INTRODUCTION

In diseases associated with oxidant stress, the balance between NO and $O_2^{\cdot-}$ in the vascular wall is likely to be perturbed, which can contribute to an insulin resistance state. The enhanced vascular levels of $O_2^{\cdot-}$ present in disease can directly damage the vasculature itself, and additionally can inactivate NO (Gryglewski *et al.*, 1986) leading to the formation $ONOO^-$ – thus promoting further endothelial dysfunction. The balance of free radicals in the vasculature is finely tuned so that any excess or shortage will lead to dysfunction (Fig. 6.1).

The relationship between oxidant stress, endothelial function and insulin resistance in the obese Zucker rat remains unclear. However, using a number of structurally diverse antioxidants added to the diet, and pro-oxidant administration it was possible to modulate the oxidant status and measure any concomitant changes in endothelial function and insulin resistance. In insulin sensitive tissues, oxidative stress associated with diabetes mellitus is likely to impair insulin action and depress endothelial function, promoting insulin resistance and cardiovascular complications. Indeed, defective endothelial function at insulin sensitive sites may contribute to poor insulin-stimulated glucose disposal (Baron *et al.*, 1995). This chapter investigates in the obese Zucker rat endothelial function, insulin resistance and oxidant stress, and their responses to dietary antioxidants (probucol, vitamin E and tiron) and pro-oxidants (HQ and BSO).

Vitamin E consists largely of alpha tocopherol (Fig. 6.2). It is considered an essential nutritional element, although its exact function is unknown. As a lipophilic antioxidant, vitamin E protects polyunsaturated fatty acids in membranes and other cellular structures from attack by free radicals, and protects red blood cells against haemolysis. Vitamin E acts as a chain breaking antioxidant that defends the cell against ROS by scavenging radicals

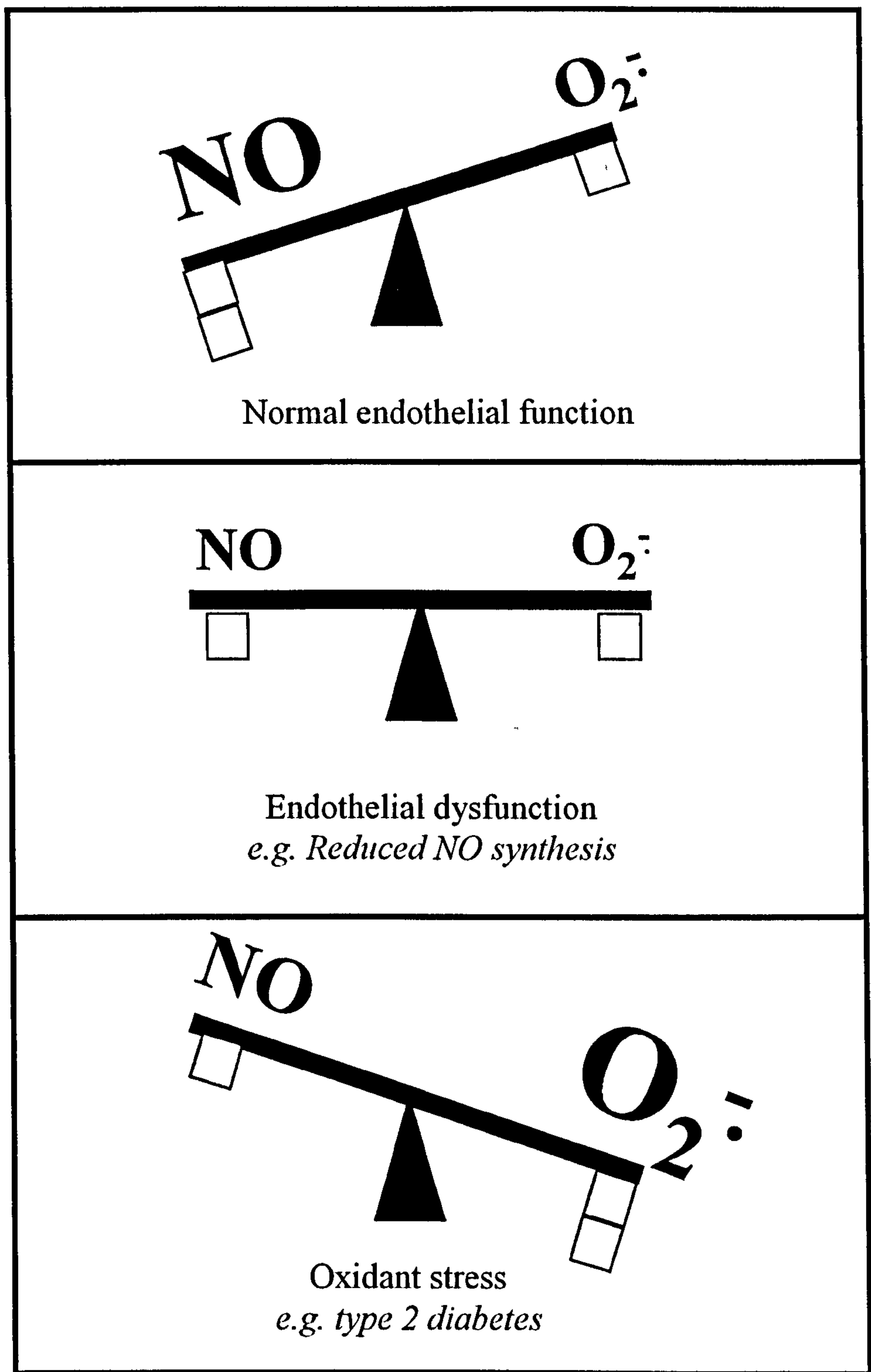


Figure 6.1 Representation of the balance between nitric oxide (NO) and superoxide anion (O₂⁻) in the vascular wall: disturbances in disease.

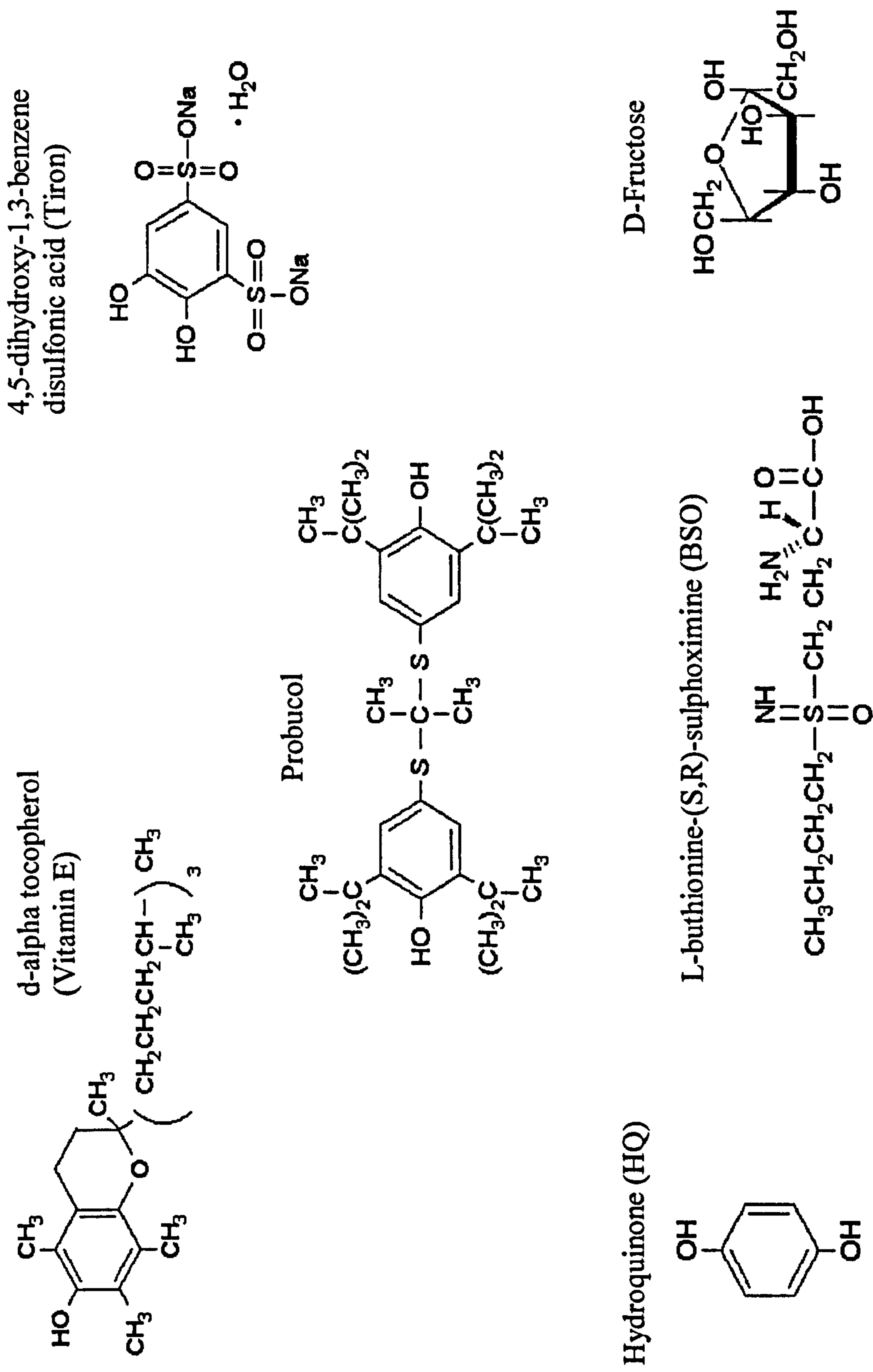
present in membranes and lipoproteins. It does this by binding and capturing peroxy radicals (chain reaction intermediates) and thus breaking the chain reaction that causes lipid peroxidation. It should be noted that protection against oxygen radical damage appears to be important for the development and maintenance of nerve and muscle function. Vitamin E may also act as a cofactor in some enzyme systems. It is bound to beta lipoproteins in blood and stored in all body tissues, especially fatty tissues. Although a large number of claims for vitamin E have been made, it has not been proven effective as a treatment for, or preventative measure against, diabetes or arteriosclerosis. Studies have shown reductions in 8-epi-PGF_{2α} and 11-dehydro-thromboxane B₂ to normal levels with vitamin E supplementation in type 2 diabetic patients (Davi *et al.*, 1999). Similarly, Vitamin E supplementation was found to effectively decrease both serum C-reactive protein and interleukin-6 in both type 2 diabetics and healthy controls (Devaraj & Jialal, 2000). In another study, type 1 diabetic children were given vitamin E, which resulted in increased glutathione levels and decreased malondialdehyde to normal values. It was concluded that antioxidant supplementation may help lower the risk of vascular disease in type 1 diabetic patients (Jainn *et al.*, 2000). Similar studies in type 2 diabetic patients have shown vitamin E treatment increased levels of glutathione, whilst decreasing free radical by-products substantially (Sharma *et al.*, 2000). More interestingly, studies have shown that pharmacological doses of vitamin E can improve insulin action in type 2 diabetics (Paolisso *et al.*, 1993).

Probucol (Fig. 6.2) is a cholesterol lowering drug with antioxidant properties. It has been shown that probucol may inhibit the oxidation and tissue deposition of LDL cholesterol, thereby slowing atherogenesis (Parthasarathy *et al.*, 1986). It has been used clinically as an adjunct to dietary measures in patients with primary hypercholesterolemia. Probucol is a dimer of hydroxytoluene, which is an antioxidant itself. Probucol's antioxidant properties stems from its two hydroxyl groups that allow it to reduce free radicals into a non-reactive

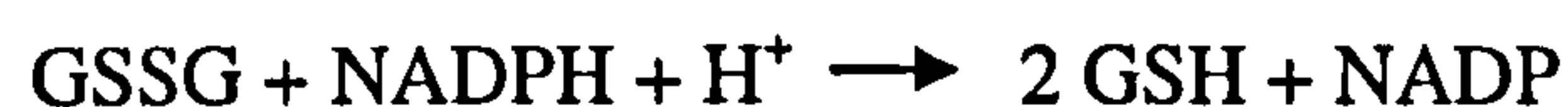
form. It also acts as a lipophilic chain breaking antioxidant. It has been demonstrated that probucol is able to protect LDL from oxidation (Parthasarathy *et al.*, 1986). Probucol has also been found to inhibit atherosclerotic lesion formation in hyperlipidaemia rabbits, through its antioxidant properties rather than its lipid lowering properties (Steinberg *et al.*, 1988). In human clinical studies, antioxidant supplementation is related to decreased oxidation of LDL and decreased manifestation of atherosclerosis. However, these effects may not be due to inhibition of the initiating events of atherosclerosis (Diaz *et al.*, 1997). Post mortems of patients who suffered from atherosclerosis indicate that atherosclerotic lesions were established in childhood (Stary, 1989). Consequently, antioxidants are thought to slow the development of atherosclerotic lesions, rather than inhibit their formation.

Tiron (fig. 6.2), a water-soluble scavenger of $O_2^{\cdot-}$, was also used, since increased $O_2^{\cdot-}$ contributes to impaired NO mediated relaxation; many studies have shown that tiron both improves vascular endothelial function (Arimura *et al.*, 2001; Didion *et al.*, 2002) and decreases the generation of $O_2^{\cdot-}$ *in vivo* (Laight *et al.*, 1998).

Pro-oxidants are compounds that induce oxidative stress by spontaneous or enzymic generation of ROS, or by inhibition of antioxidants. Hydroquinones belong to the quinone class of redox cycling compounds that undergo one electron reductions in the presence of enzymes such as mitochondrial NADH, microsomal NADPH and flavoenzymes. Quinone redox cycling is associated with rapid conversion of O_2 to $O_2^{\cdot-}$, and therefore this class of reagents are useful pro-oxidant research compounds. Previous studies have shown HQ to blunt the NO vasorelaxant responses in many different animal tissues such as aortic strips (Moncada *et al.*, 1986), rabbit coronary arteries (Bing *et al.*, 1987) and cat cerebral arteries (Alonso *et al.*, 1992).



BSO is a potent, selective and irreversible inhibitor of GSH synthetase (Griffith, 1999). Glutathione is a sulphur containing compound that is an essential co-factor for many antioxidant enzymes in the body, including glutathione peroxidase. Higher levels of glutathione are desirable, indicating a larger supply for the body to incorporate into antioxidant enzymes. GSH is a very effective reducing agent and an important scavenger of ROS. It is present in the mitochondria, endoplasmic reticulum and nucleus. The liver is the main source of GSH synthesis, from where it can be transported via the blood to the tissues (Gamalay & Klyubin, 1999). Glutathione peroxidase reduces hydroperoxides in a reaction that generates oxidized GSH (glutathione disulfide, GSSG), which is subsequently recycled to GSH by glutathione reductase.



Glutathione maintains Fe^{3+} and cysteinyl residues in haemoglobin in the reduced state and reacts rapidly with OH^\bullet , ONOO^- , and $\text{O}_2^{\bullet-}$. Studies in animals suggest that vitamin E supplementation helps increase glutathione levels, possibly by assisting in the neutralization of free radicals, thus decreasing the oxidative stress on the body. BSO decreases GSH levels in the kidney, liver, pancreas, muscle and plasma, and evokes increased levels of ROS. It has been demonstrated that 5 hours after the administration of BSO, GSH was reduced by 40 % in liver samples of treated rats. Studies showed no measurable affect by BSO in rat aortic rings (Cuzzocrea *et al.*, 1998; Laight *et al.*, 1999C). Although not capable of altering vasoregulation in its own right, BSO has been shown to aggravate the detrimental effect of HQ on vasodilation in rat aortic rings (Laight *et al.*, 1999).

Markers of oxidant stress are raised in type 2 diabetes, which is indicative of increased levels of ROS (Gopaul *et al.*, 1995). Furthermore, a reduction of antioxidant defences has

also been observed in both diabetic patients (Collier *et al.*, 1990; Vijayalingam *et al.*, 1996) and pre-diabetic rats (Gunnarsson *et al.*, 1998). The marker of oxidant stress, 8-epi-PGF_{2α}, was used in these studies to establish the antioxidant or pro-oxidant action *in vivo* of the drugs used and to compare the changes in 8-epi-PGF_{2α} levels with changes in diabetic pathology. It is known that increased oxidant stress causes cell damage (Halliwell, 1994) and even cell death. Increased oxidant stress in type 2 diabetes may contribute to its complications and, more importantly, be involved in the primary onset of the disease. By using end products of lipid peroxidation, a number of methods can be employed to assess oxidative stress in plasma and tissue.

6.2 RESULTS

6.2.1 Effect of antioxidants on endothelial function, oxidant stress and insulin resistance in the Zucker rat.

Plasma insulin and glucose levels are altered by antioxidant diets. Obese basal plasma glucose and insulin levels were elevated relative to lean levels ($P<0.05$), and were slightly reduced after dietary vitamin E, tiron and probucol ($P>0.05$) (Fig. 6.3). In contrast, basal lean plasma glucose levels was elevated by dietary vitamin E, ($P<0.05$) but not tiron or probucol. Basal lean insulin levels were not affected ($P>0.05$) (Fig. 6.3).

Following bolus glucose ($0.5 \text{ g kg}^{-1} \text{ i.v.}$) administration, both the plasma glucose and insulin responses were greater in obese animals (Fig. 6.4). Dietary vitamin E in obese animals reduced insulin AUC ($P<0.05$), without an effect on glucose AUC ($P>0.05$). In contrast, dietary vitamin E, tiron and probucol appeared to elevate glucose and insulin AUC in lean animals. Although statistical significance was not attained, more n numbers may have given significance. Comparisons of the insulin/ glucose ratio showed a similar profile to that of insulin AUC – impairment in glucose disposal in obese compared to lean rats, and improvement with antioxidant treatment in obese but not lean groups (Fig. 6.4C).

Antioxidant diet had no effect on body weight, however the obese rats were significantly heavier than the lean animals ($P<0.05$) (table 6.1).

Antioxidant diet had no effect on basal and Phe -induced PP. Basal PP in obese and lean preparations were not affected by dietary vitamin E, tiron or probucol ($P>0.05$) (table 6.2). Similarly, Phe-elevated perfusion pressures in obese and lean preparations were not significantly altered by dietary vitamin E, tiron or probucol ($P>0.05$) (table 6.2).

Treatment	control	Vitamin E	tiron	Probucol
Obese	413.6±15.2*	428.2±8.6*	448.2±10.7*	455.2±9.3*
Lean	280.2±4.2	280.6±6.3	282.2±17.3	305.0±7.4

Table 6.1 Body weights (g) of lean and obese Zucker rats following 4 weeks of dietary treatment. Data are expressed as mean±s.e.mean of 3-6 determinations. **P*<0.05 with respect to corresponding treatment in the lean group.

(mm Hg)	Treatment			
Basal PP	control	Vitamin E	tiron	Probucol
Obese	28.5±6.1	29.9±5.4	32.4±2.7	26.18±2.7
Lean	38.1±5.8	25.5±2.3	24.4±2.0	30.6±2.0
Phe-elevated PP	control	Vitamin E	tiron	Probucol
Obese	144.2±20.5	144.8±13.0	113.6±16.5	132.0±5.0
Lean	129.6±6.0	141.6±13.3	126.6±7.5	142.1±15.2

Table 6.2 Basal and Phe elevated PP in lean and obese Zucker rats following dietary treatments. Data are expressed as mean±s.e.mean of 3-6 determinations.

Endothelial hyperactivity is altered by antioxidant diets. Vasodilator responses to ACh (0.0001-10 nmol) were enhanced in obese relative to lean animals (*P*<0.05 2W ANOVA) (Fig. 6.5), while *pD*₂ and *E*_{max} values were similar in the obese and lean Zucker rats (*P*>0.05). Similarly, the maximal vasodilation to SNP (100 nmol) was comparable between lean and obese (table 6.3).

Comparison between treatment groups showed that vitamin E significantly blunted vasorelaxation in both the lean and obese rats (Fig. 6.5A; 6.5B; table 6.4). This effect was associated with a decrease in the magnitude of vasodilator responses to ACh (0.0001-10 nmol) in obese (*P*<0.05 2W ANOVA) compared to the level of vasorelaxation in the lean hindquarters preparations (*P*>0.05 2W ANOVA). Dietary treatment with tiron (Fig. 6.5C;

6.5D) and probucol (Fig. 6.5E; 6.5F) both induced a greater vasodilation in the lean and obese rats. Significance was reached by probucol in the obese group, and by tiron in lean group ($P<0.05$, 2W ANOVA).

	Treatment			
E_{\max} (%)	control	Vitamin E	tiron	Probucol
Obese	37.2±3.6	45.3±6.1	41.2±2.6	41.7±1.7
Lean	41.5±3.0	48.1±6.5	37.4±4.0	40.5±1.9

Table 6.3 Vasodilation to SNP (100 nmol) following dietary treatment in lean and obese Zucker rats. Data are expressed as mean±s.e.mean of 3-6 determinations.

In contrast to endothelium-dependent responses, maximal vasodilation to SNP was not significantly enhanced ($P>0.05$) following 4-week dietary vitamin E, and unchanged in tiron and probucol obese and lean hindquarters preparations. Similarly, using pD_2 and E_{\max} values, comparison of the curves were not significantly different in either the control or treatment groups.

	Treatment			
E_{\max} (%)	control	Vitamin E	tiron	Probucol
Obese	134.6±6.7	105.1±5.8	123.8±5.3	148.8±9.6
Lean	115.5±5.3	84.3±9.4	127.7±16.5	121.7±9.6

pD_2	control	Vitamin E	tiron	Probucol
Obese	10.37±0.07	10.34±0.15	10.75±0.12	10.55±0.07
Lean	10.39±0.07	10.21±0.21	10.57±0.13	10.39±0.16

Table 6.4 Effect of 4-week dietary vitamin E, tiron or probucol on vasodilation to ACh (0.0001-10 nmol) standardised with respect to vasodilation to SNP (100 nmol) in the perfused hindquarters *in situ* of obese and lean Zucker rats. Data are expressed as mean±s.e.mean of 3-6 determinations.

Lower 8-epi-PGF_{2α} plasma levels in antioxidant treated Zucker rats. Plasma levels of 8-epi-PGF_{2α} were increased (by about 5 fold) in the obese relative to the lean ($P<0.05$) Zucker rats (Fig. 6.6). Treatment with Vitamin E and probucol but not tiron, decreased plasma 8-epi-PGF_{2α} levels in obese ($P<0.05$) to levels comparable with their lean littermate. Dietary antioxidant treatments showed no effect in the lean ($P>0.05$) Zucker rats.

6.2.3 Effect of pro-oxidants on endothelial function, oxidant stress and insulin resistance in the Zucker rat.

Plasma insulin and glucose levels are altered by pro-oxidant treatment. Obese basal plasma insulin levels were elevated relative to the lean ($P<0.05$). However, the lean groups treated with HQ and BSO alone showed a further decrease in insulin levels compared to the control rats (Fig. 6.7A). Basal plasma glucose levels were comparable in the lean and obese rats. However, pro-oxidant treatments with HQ, BSO and HQ+BSO caused an increase in the levels of glucose when compared with lean ($P<0.05$) (Fig. 6.7B).

Following bolus glucose (0.5 g kg^{-1} i.v.) administration, plasma insulin responses were greater in untreated obese animals compared to the lean rats; although statistical significance was not reached, possibly due to a large variation between animals in the group – again more n numbers may have reduced this variation ($P>0.05$) (Fig. 6.8A). Pro-oxidant treatments slightly increased insulin AUC in the obese groups to cause a difference in the lean and obese treatment groups when compared individually ($P<0.05$ students t -test).

Plasma glucose was significantly elevated in the obese compared to the lean rats. However, treatment with pro-oxidants decreased the obese glucose levels to a level comparable with

their lean treatment equivalent (Fig. 6.8B). Comparisons of the insulin/glucose ratio showed slightly higher levels in the obese compared to the lean. Though HQ+BSO treatment increased the obese ratio significantly. Differences were also significant in the lean and obese treatment groups HQ and HQ+BSO (Fig. 6.8C).

Pro-oxidant treatment had no effect on body weights of 13 week-old obese Zucker rats. Lean and obese rats were not affected by pro-oxidant treatments. Obese rats were greater ($P<0.05$) than that of the corresponding lean Zucker rat weights (table 6.5).

Treatment	Control	HQ	BSO	HQ+BSO
Obese	432.7±10.7*	421.4±14.2*	433.2±6.7*	413.5±10.8*
Lean	270.0±11.1	260.0±6.6	277.0±10.4	260.5±4.6

Table 6.5 Body weights (g) of lean and obese Zucker rats following pro-oxidant treatment. Data are expressed as mean±s.e.mean of 4-6 determinations. * $P<0.05$ with respect to corresponding lean group.

Pro-oxidant treatment had no effect on basal and Phe-induced PP. Basal PP was not affected by pro-oxidant treatments in obese preparations ($P>0.05$) (table 6.6). Similarly, lean basal PPs were comparable to the obese, and were unaffected by pro-oxidant treatment ($P>0.05$). Phe-induced PP in obese preparations were not affected by pro-oxidant treatments ($P>0.05$). Lean and obese basal PPs were comparable and unaffected by pro-oxidant treatment ($P>0.05$) (table 6.6).

Endothelial hyperactivity is impaired by pro-oxidant treatment. Vasodilator responses to ACh (0.0001-10 nmol) were enhanced ($P<0.05$) in obese relative to lean animals (see Fig. 3.11; table 6.7). This difference was abolished following pro-oxidant treatment with HQ, BSO, and HQ+BSO ($P>0.05$) (Fig. 6.9; table 6.7). The abolition of the ACh-induced hyperreactivity in the obese was associated with both a decrease in the magnitude of

vasodilation when treated with HQ ($P<0.05$) and HQ+BSO ($P<0.05$), and with small but significant increases in lean responses (HQ: $P<0.05$; HQ+BSO: $P<0.05$). BSO alone did not change the responses in lean or obese rats ($P>0.05$).

(mm Hg)	Treatment			
Basal PP	Control	HQ	BSO	HQ+BSO
Obese	27.7±3.1	33.5±3.9	30.3±8.8	35.8±4.5
Lean	31.2±2.5	31.3±3.8	46.4±9.6	28.2±1.5
Phe-elevated PP	Control	HQ	BSO	HQ+BSO
Obese	140.8±18.3	176.5±11.0	134.9±18.1	167.7±15.6
Lean	175.0±13.5	201.5±23.2	209.2±54.1	165.7±22.6

Table 6.6 Effect of pro-oxidant treatment on basal and Phe-elevated PP in lean and obese Zucker rat perfused hindquarters *in situ*. Data are expressed as mean±s.e.mean of 4-6 determinations.

	Treatment			
E _{max} (% SNP)	Control	HQ	BSO	HQ+BSO
Obese	132.6±6.3*	121.6±5.5	127.2±12.3	107.1±3.4†
Lean	111.9±3.6	120.2±8.9	114.4±3.7	115.8±2.5
pD ₂	Control	HQ	BSO	HQ+BSO
Obese	10.95±0.06*	10.70±0.02† ^ψ	11.07±0.04	10.74±0.01† ^ψ
Lean	10.69±0.01	10.59±0.01	10.58±0.02	10.81±0.02

Table 6.7 Effects of pro-oxidant treatment on vasodilation to ACh (0.0001-10 nmol) standardised with respect to vasodilation to SNP (100 nmol) in the perfused hindquarters *in situ* of obese and lean Zucker rats. Data are expressed as mean±s.e.mean of 4-6 determinations. * $P<0.05$ with respect to corresponding lean group; † $P<0.05$ with respect to obese control; ^ψ $P<0.05$ with respect to lean control.

Higher 8-epi-PGF_{2α} plasma levels in pro-oxidant treated Zucker rats. Plasma levels of 8-epi-PGF_{2α} were increased in the obese (5.46 ± 1.0 ng mL⁻¹, $n=6$) relative to the lean (2.70 ± 0.3 ng mL⁻¹, $n=6$; $P<0.05$) Zucker rats (Fig. 6.10). Pro-oxidant treatment increased plasma 8-epi-PGF_{2α} levels compared to untreated in obese ($P<0.05$) but not lean ($P>0.05$) Zucker rats.

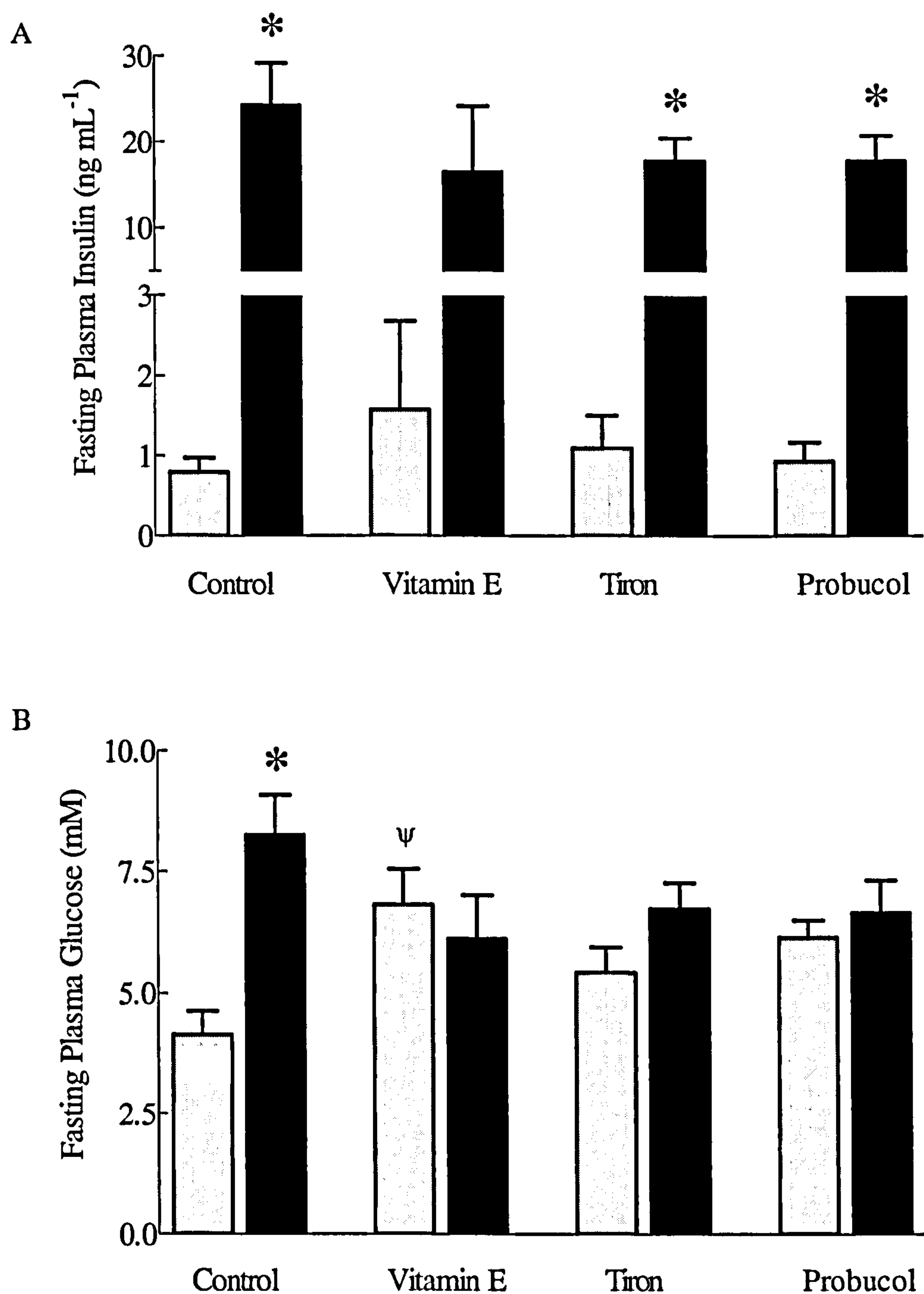


Figure 6.3 Measurement of basal plasma insulin (ng mL⁻¹) and glucose (mM) in 13 week-old lean (■) and obese (■) Zucker rats treated with sham, Vitamin E, Tiron and Probucol. Data are expressed as mean±s.e.mean of 5-6 determinations. Statistical significance was compared using a student t-test, * $P < 0.05$ with respect to the equivalent lean control group, or using one way ANOVA, ^Ψ $P < 0.05$ with respect to the control treatment group.

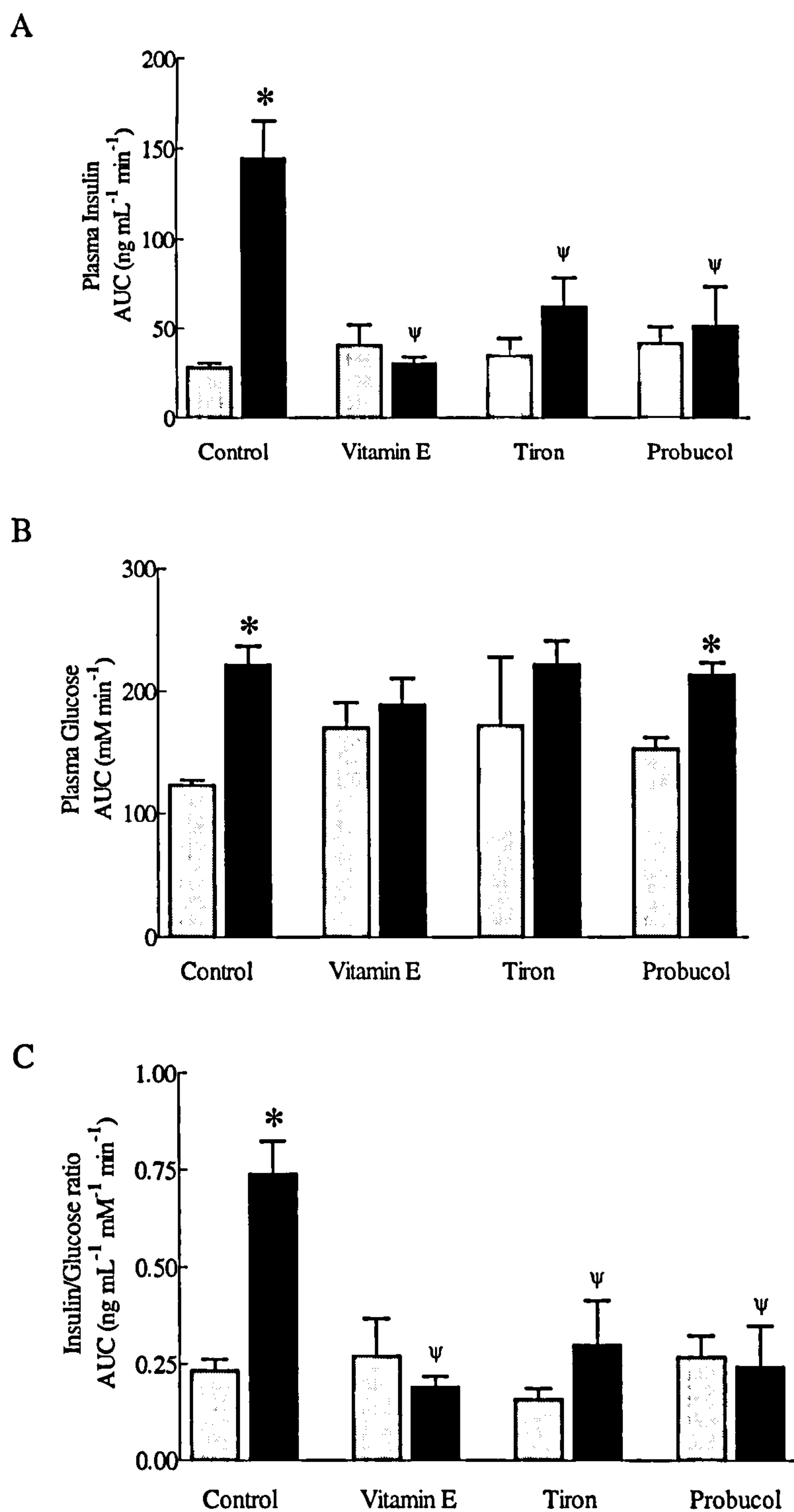


Figure 6.4 Glucose tolerance test (g.t.t) in 13 week-old lean (■) and obese (■) Zucker rats following a 4 week dietary treatment of vitamin E, Tiron or probucol. A. Comparison of plasma insulin (AUC, 0-24 min). B. Plasma glucose (AUC, 0- 24 min). C. Insulin/Glucose ratio (AUC, 0-24 min). Data are expressed as mean±s.e.mean of 3-6 determinations. Statistical significance was compared using a student t-test, * $P<0.05$ with respect to the equivalent lean control group, or using one way ANOVA, $^{\Psi}P<0.05$ with respect to the equivalent lean control group.

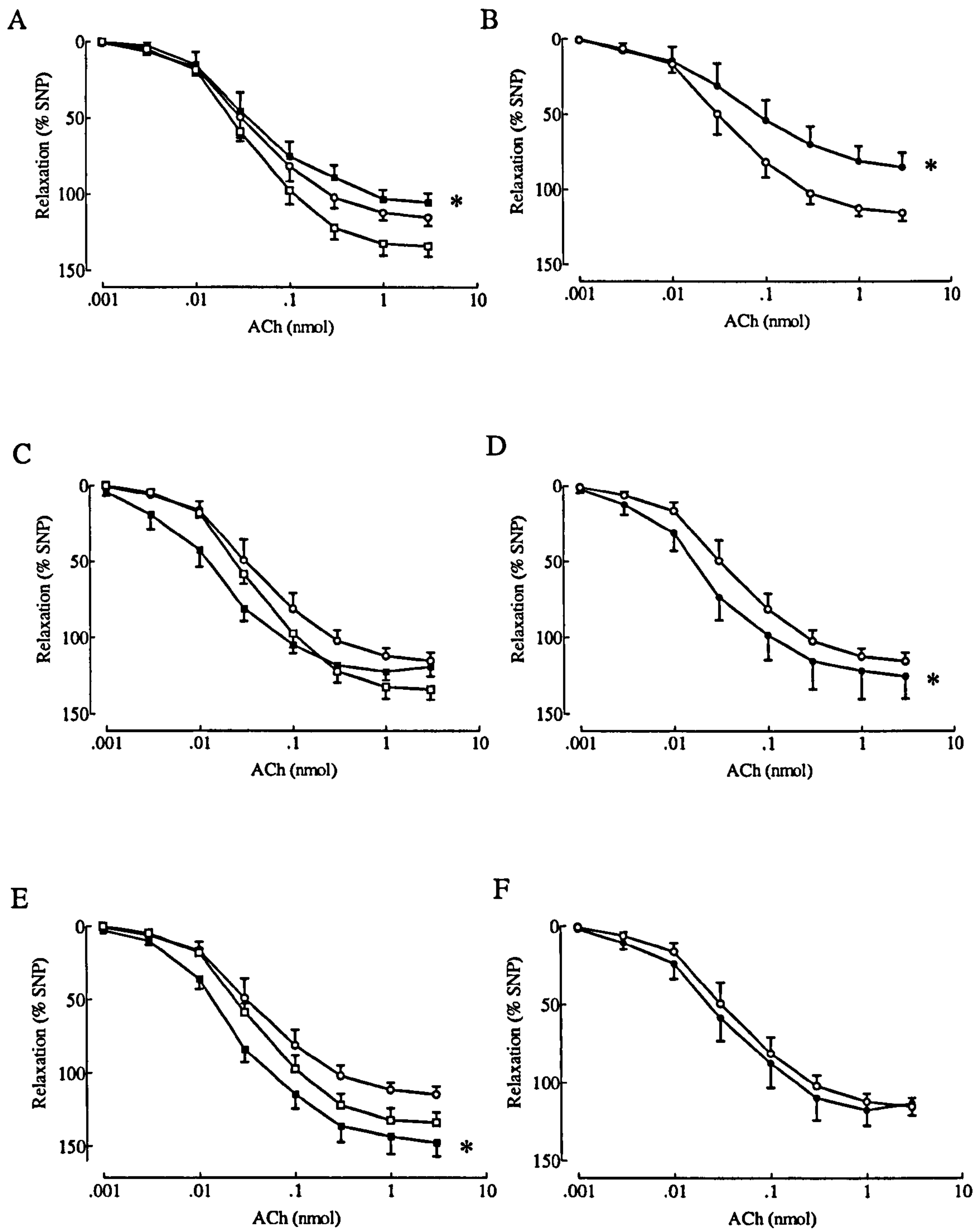


Figure 6.5 Effects of 4 week dietary anti-oxidant treatment on vasodilation to acetylcholine (ACh, 0.001-10 nmol) in 13-week old lean and obese Zucker rats perfused hindquarters *in situ*. Control lean (○) and obese (□) rats are compared to lean (●) and obese (■) treatment groups: A-B, vitamin E, C-D, tiron and E-F, probucol. ACh responses were standardised with respect to vasodilation to sodium nitroprusside (SNP, 100 nmol). Data are expressed as mean±s.e.mean of 3-6 determinations. Statistical significance (*) using 2 way ANOVA was considered established at $P < 0.05$.

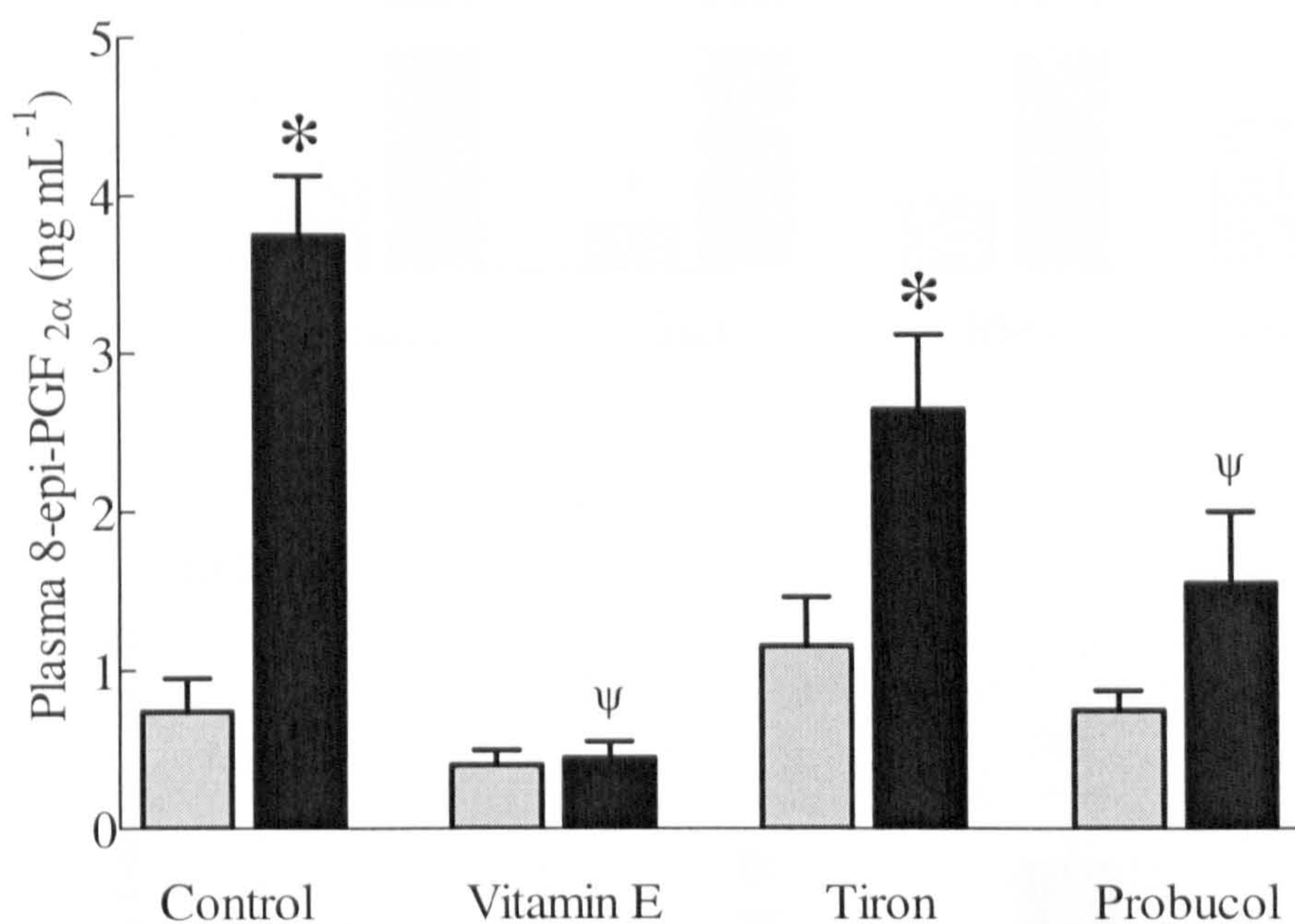


Figure 6.6 Plasma levels of 8-epi-prostaglandin $F_{2\alpha}$ (8-epi-PGF $_{2\alpha}$) in lean (■) and obese (■) Zucker rats: effects of 4 week dietary treatment with vitamin E, tiron or probucol. Data are expressed as mean \pm s.e.mean of 4-6 determinations. Statistical significance was compared using a student t-test, * $P<0.05$ with respect to lean control group, or using one way ANOVA, $^{\psi}P<0.05$ with respect to obese control group.

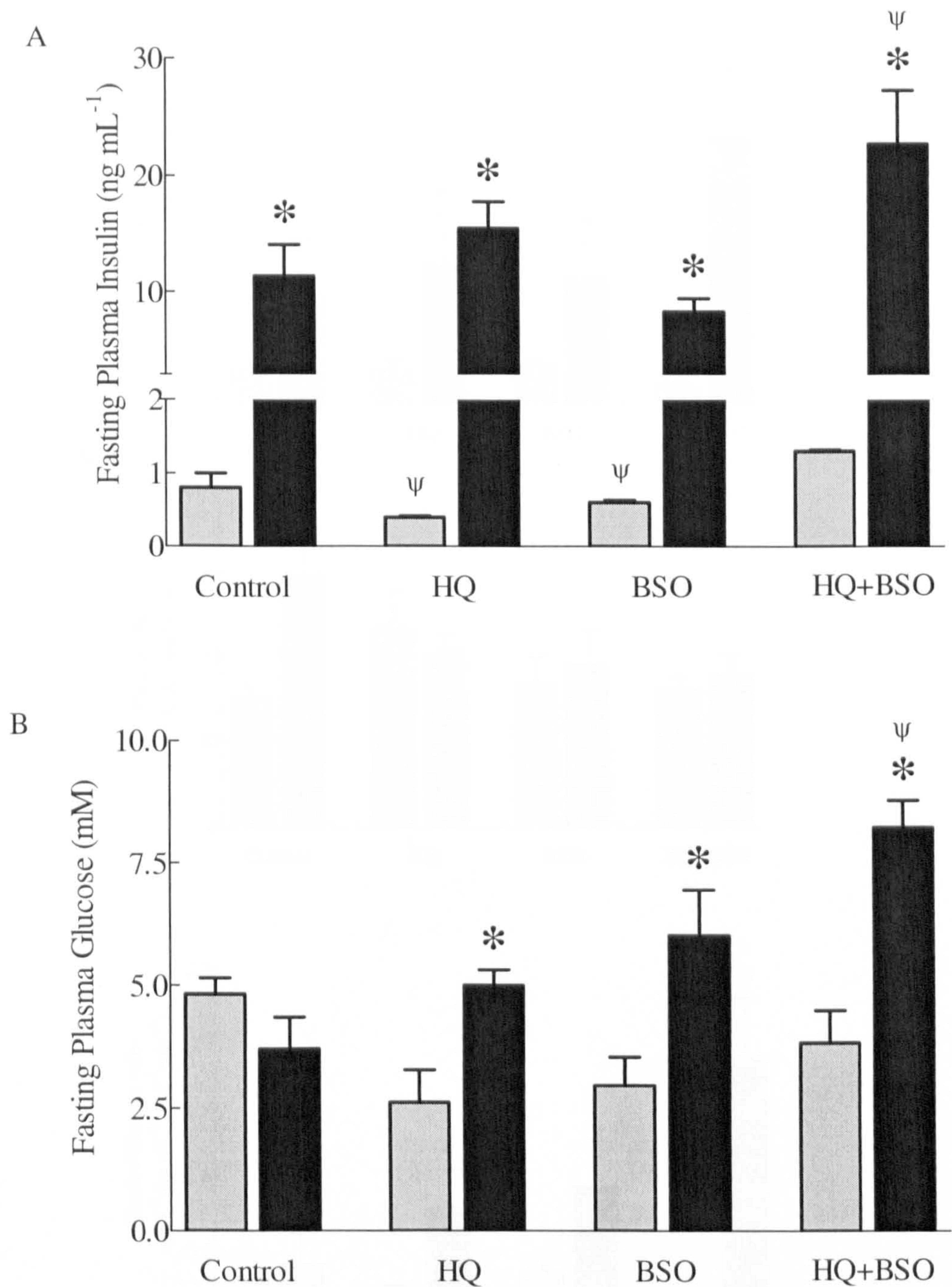


Figure 6.7 Measurement of basal plasma insulin (ng mL⁻¹) and glucose (mM) in 13 week old lean (■) and obese (■) Zucker rats treated with sham, HQ, BSO and HQ+BSO in combination. Data are expressed as mean±s.e.mean of 4-6 determinations. Statistical significance was compared using a student t-test, * $P < 0.05$ with respect to the equivalent lean control, or using one way ANOVA, ^Ψ $P < 0.05$ with respect to the control treatment group.

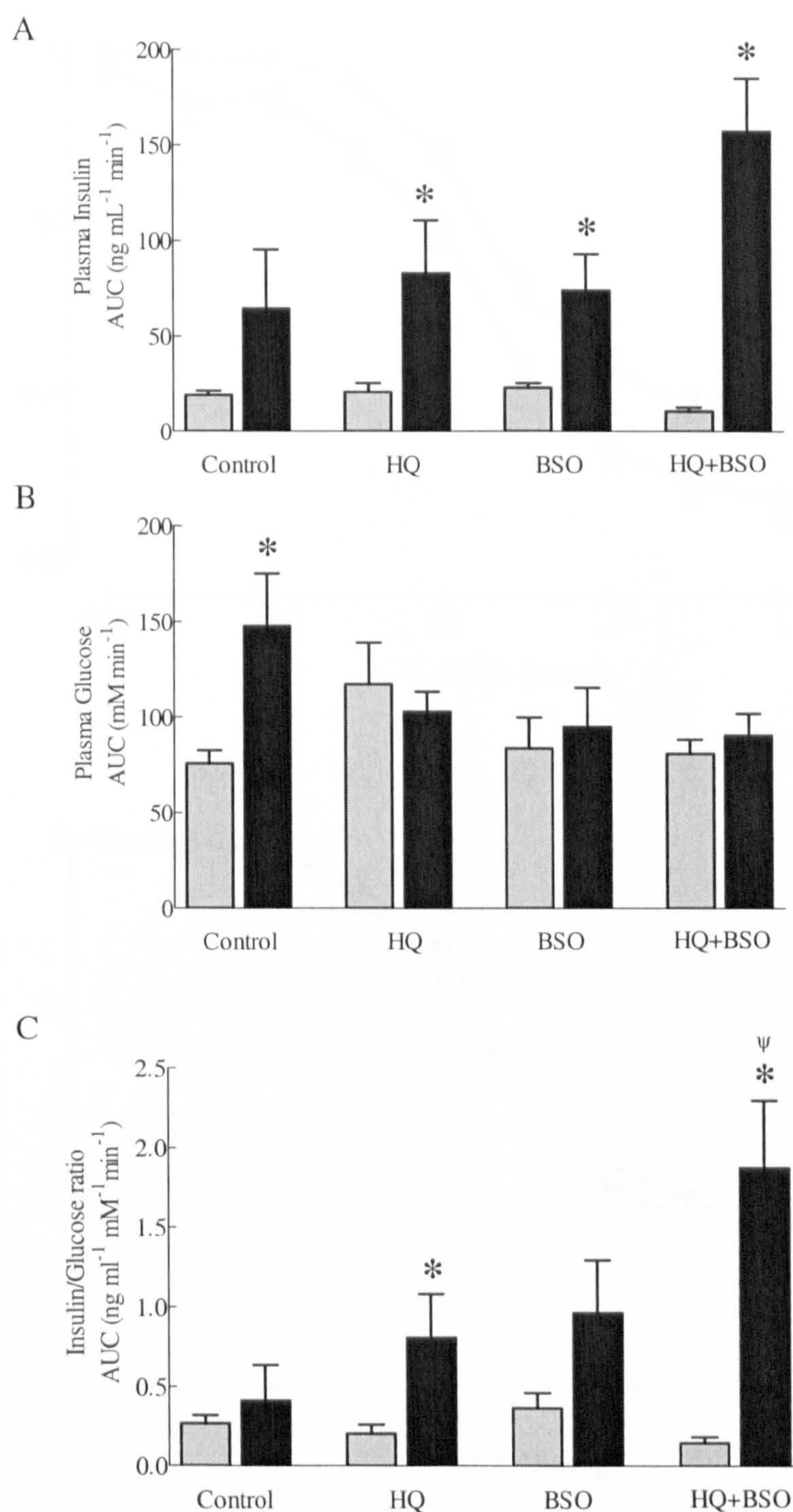


Figure 6.8 Glucose tolerance test (g.t.t) in 13 week-old lean (■) and obese (■) Zucker rats following a one week treatment of hydroquinone and buthionine sulfoxamine combination (HQ+BSO) (50 mg kg⁻¹ i.p. daily). A. Comparison of plasma insulin (AUC, 0-24 min). B. Plasma glucose (AUC, 0- 24 min). C. Insulin/Glucose ratio (AUC, 0-24 min). Data are expressed as mean±s.e.mean of 4-6 determinations. Statistical significance was compared using a student t-test, **P*<0.05 with respect to the equivalent lean control group, or using one way ANOVA, ^Ψ*P*<0.05 with respect to the equivalent control treatment group.

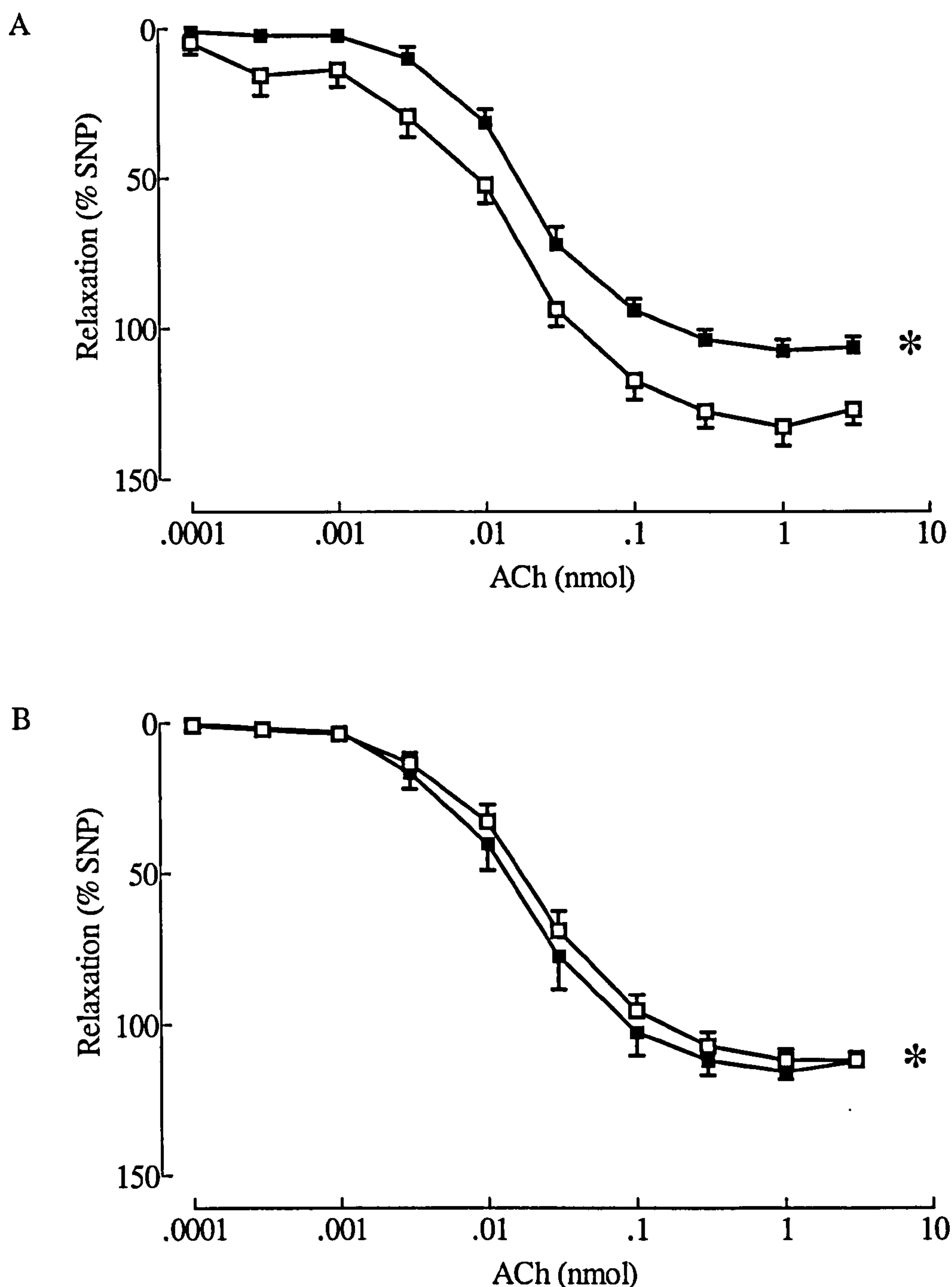


Figure 6.9 Effects of hydroquinone and buthionine sulfoxamine combination (HQ+BSO) (50 mg kg⁻¹ i.p. daily) for one week on vasodilation to acetylcholine (ACh, 0.001-10 nmol) in 13 week-old lean and obese Zucker rat perfused hindquarters *in situ*. Comparison of A. obese B. lean control (□) and obese HQ+BSO (■) treated. ACh responses were standardised with respect to vasodilation to sodium nitroprusside (SNP, 100 nmol). Data are expressed as mean±s.e.mean of 4-6 determinations. Statistical significance using two way ANOVA was **P*<0.05.

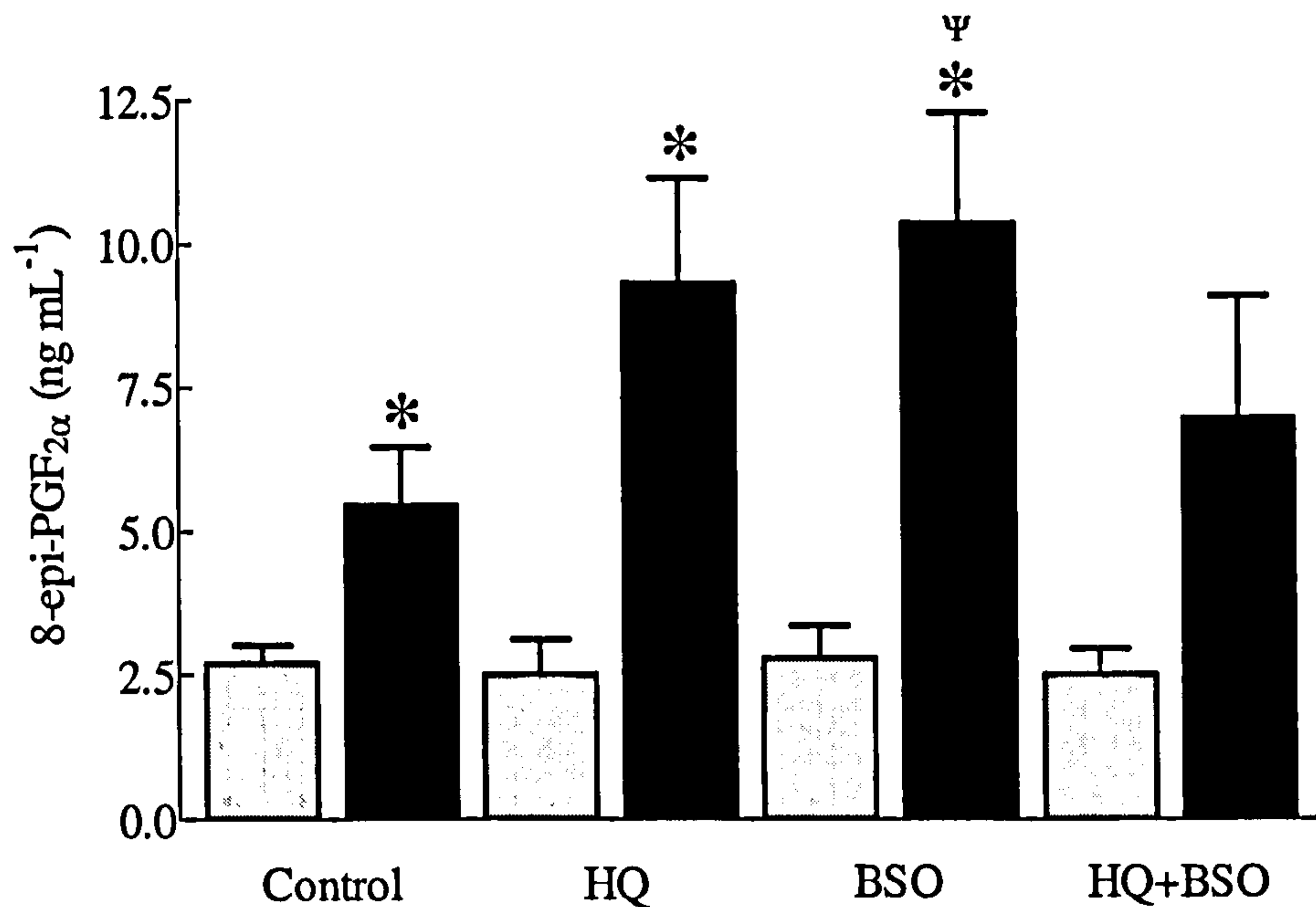


Figure 6.10 Plasma levels of 8-epi prostaglandin F_{2α} in 13 week-old lean (□) and obese (■) Zucker rats treated with sham, hydroquinone (HQ) and buthionine sulfoxamine (BSO) alone or in combination (HQ+BSO) (50 mg kg⁻¹ i.p. daily). Data are expressed as mean±s.e.mean of 6 determinations. Statistical significance was compared using a student t-test, **P*<0.05 with respect to lean control group, or using one way ANOVA, Ψ*P*<0.05 with respect to obese control group.

6.3 DISCUSSION

Antioxidant treatment The elevated plasma insulin and glucose levels seen in the obese compared to the lean rats were not reduced with antioxidant treatment. However, in response to a glucose load, the elevated insulin levels in the obese rats were decreased by antioxidant treatment. The responses in the lean animals were unaffected by antioxidant treatment. The plasma insulin levels from the obese rats were elevated to an unexpected level (24 ng mL^{-1}) compared ($6\text{-}12 \text{ ng mL}^{-1}$) seen in the previous studies. The increased insulin levels were only seen in the obese rats and may be due to animal variation and not insulin analysis or plasma storage. The result do show relative differences between the treatment groups and therefore the data can provide useful understanding of the action of antioxidant treatment in this particular batch of animals. These results would imply that the obese rats have higher basal insulin levels, necessary to overcome the increased glucose ingested due to their hyperphagia. The increased basal insulin level is sufficient to maintain relatively normal plasma glucose levels, which are comparable to its lean littermate. However, following a glucose load, the obese rat produced relatively more insulin to dispose of the glucose administered, thus revealing insulin resistance. Antioxidant treatment decreased the need for this greater production of insulin in the obese rat, perhaps by improvement in endothelial function at the skeletal muscle.

The enhanced endothelium-dependent vasodilation in the obese compared to the lean Zucker rat was abolished following 4-week dietary vitamin E. Plasma 8-epi-PGF_{2α} levels were decreased in the obese Zucker rat. This data suggests the increased compensatory endothelium-dependent vasodilation was no longer required following vitamin E treatment. As the dietary vitamin E treatment did not enhance vasodilation to SNP, it can be understood as an endothelium-dependent effect. This is in agreement with other studies where Vitamin E prevented impairment of the vascular endothelium, and even reversed

endothelial dysfunction in animal models of vascular disease (Stewart-Lee *et al.*, 1994; Andersson *et al.*, 1994; Laight *et al.*, 1996; Konneh *et al.*, 1995; Laight *et al.*, 1997). This antioxidant action on endothelial function may be due partly to the protection of endothelium-derived NO from inactivation by ROS (Gryglewski *et al.*, 1986; Laight *et al.*, 1998), and partly that agonist-stimulated endothelial vasodilator function is modulated by oxidant tone differently in lean and obese Zucker rats. In any event, the apparent need for endothelial hyperfunction in the obese was removed by vitamin E. Another explanation for the observed effect may be that ROS cause a net upregulation of agonist-stimulated endothelium-dependent vasodilation in the obese Zucker rat, whilst inactivating the NO that is subsequently synthesised. It has been demonstrated that oxidative stress may affect specific receptors and intracellular signalling cascades (Flavahan, 1992; Stewart-Lee *et al.*, 1994, 1995). The upregulation of ROS is certainly an interesting speculation, as leptin has been shown to cause direct vasodilation through an endothelium-dependent mechanism (Lembo *et al.*, 2000), as well as to increase oxidative stress in human endothelial cells. It may be that ROS are second messengers in leptin-induced signalling in endothelial cells, and contribute to the direct endothelium-dependent vasodilation by leptin. Furthermore, hyperleptinaemia may contribute to chronic oxidant stress in endothelial cells and to vascular pathology (Bouloumie *et al.*, 1999). It is important to note that while an upregulation in the synthesis/release of endothelium-derived relaxing factors such as prostacyclin or NO cannot be ruled out in the adaptive response to this elevated oxidant tone, there may also be a non-prostanoid, non-NO component of vasodilation. In this respect, a possible hyperpolarising factor-derived mechanism has been described in the isolated aorta of the obese Zucker rat (Kaw *et al.*, 1999).

In contrast, endothelium-dependent vasodilation was significantly improved by probucol but not by tiron. Furthermore, the measured oxidant stress correlated with the endothelial function in that probucol, but not tiron, significantly decreased plasma 8-epi-PGF_{2α}. These

results are somewhat contrary to the reduced vasodilation seen with vitamin E treatment. However, previous studies by Keaney *et al.*, (1994; 1995) have also shown vitamin E to worsen, and probucol to preserve endothelial function in cholesterol fed rabbits. One explanation for this could be, in addition to its antioxidant activity, probucol's hypocholesterolaemic activity. This secondary role of probucol is not clearly understood but may involve either an immunomodulatory activity (Wagberg *et al.*, 2001) or Ca^{2+} blocking effect which is thought to be involved in reduction of fatty streak formation in atherosclerosis. It would seem probable that in a hyperlipidaemic Zucker rat, any slight decrease in early atheroma would increase vascular pliability. One study has even showed probucol to block a Ca^{2+} sensitive K^+ channel, an effect that would also increase vasodilation in the hindquarters (Howland *et al.*, 1984). In terms of the vascular balance hypothesis, probucol may act in a similar fashion to vitamin E, in decreasing the $\text{O}_2^{\cdot-}$ levels and thereby decreasing the need for NO compensation. However, this secondary role of probucol improves vascular function through another mechanism, where agonist stimulated endothelial-dependent vasodilation is improved even when NO levels are decreased. The lack of effect seen with tiron, shows in both endothelial function and in reduction of 8-epi- $\text{PGF}_{2\alpha}$, and is probably due to its hydrophilic nature. Reduction in lipid peroxidation is clearly via lipid soluble drugs that can enter the cell membrane and act *in situ*. However, tiron would have had some action to mop up the ROS generated in the plasma and intracellularly. Unfortunately though, tiron was not potent enough to generate a functional response in the hindquarters.

Overall, the efficacy of the antioxidants, in modulating hindquarters endothelial vasodilator hyperreactivity in the obese Zucker rat suggests that this improvement in insulin resistance is related to differences in the regulation of agonist-stimulated endothelium-dependent vasodilation by oxidant tone in lean and obese animals *in vivo*. However, in the absence of defects in endothelial-dependent function in the obese Zucker rat, either *in vivo* or *in situ*,

which may conceivably reflect a compensatory enhancement of endothelial function in the face of oxidant stress, the ability of the antioxidants treatments to affect further improvements in function is probably limited. Therefore pro-oxidant interventions were also done to raise oxidant stress in the obese Zucker rat *in vivo* and to expose endothelial dysfunction, in the hope of a better understanding of the vascular implications of oxidant stress in this model of insulin resistance.

Pro-oxidant treatment (HQ+BSO) increased plasma insulin and glucose levels in the obese Zucker rats. Glucose disposal was measured as insulin/glucose ratio (a parameter indicative of insulin sensitivity) to estimate the glucose-insulin feedback mechanism after fasting. It is similar to the widely accepted homeostasis model approach (HOMA), where the calculation: fasting insulin x fasting glucose /22.5 equals a HOMA score. This score correlates well to independent measures of insulin resistance and beta-cell function using the euglycaemic clamp technique (Bonora *et al.*, 2000). In these studies, the glucose disposal (insulin/glucose ratio) was also increased in the obese rats, suggesting a metabolic deterioration that worsens the already present insulin resistance in the obese rats.

Metabolic deterioration was accompanied by changes in endothelial function and oxidant stress. Pro-oxidant treatment with HQ and HQ+BSO abolished the hyperreactivity of endothelial-dependent responses to ACh in obese relative to lean Zucker rats. This effect in obese rats was associated with a decrease in the magnitude of vasodilator responses to ACh with HQ and HQ+BSO treatment. However, respective lean Zucker rat responses showed small but significant increases in reactivity. This abolished endothelial hyperreactivity by HQ treatment in the obese Zucker rat supports other functional studies in vascular rings that showed HQ can impair ACh-induced relaxation (Furchgott, 1984; Lubbe *et al.*, 1992), and enhance Phe-induced contraction (Kaw *et al.*, 1998). This would be expected, as HQ is known to generate $O_2^{\cdot-}$, and was also recently reported to have NO scavenging properties

(La *et al.*, 1999). Therefore increased levels of $O_2^{\cdot-}$, or even HQ itself could bind NO produced by endothelial cells, thereby reducing vasorelaxation. In addition, the increased levels of $O_2^{\cdot-}$ could react with the already high levels of NO to form $ONOO^-$ (Gryglewski, 1986), thereby promoting further endothelial dysfunction through cell damage. Indeed, as would be expected, the 8-epi-PGF_{2α} levels were also increased by pro-oxidant treatment. However, lean rats showed an increased vasoreactivity with pro-oxidant treatment. It may be that obese rats had reached their maximum oxidant status and could not counteract the excess free radical generation. For example, untreated obese Zucker rats may have elevated endogenous cellular antioxidants such as SOD, catalase, and glutathione. Indeed, previous studies have shown increased activity of catalase, glutathione peroxidase and SOD in endothelial cells following exposure to H_2O_2 , suggesting the presence of inducible antioxidant defences (Lu *et al.*, 1993). It may be that following chronic pro-oxidant treatment, these endogenous antioxidant pathways become exhausted. Experiments of cell-mediated oxidation of LDL have shown that the propagative phase of lipid peroxidation begins when more than 50 % of the initial chain-breaking antioxidants are depleted (Bowry *et al.*, 1992). Alternatively, the lean animals may display an enhanced vasorelaxation as compensation for the pro-oxidant treatment; comparable to what is seen in the control obese rats. This suggests that lean rats are able to cope with the increased turnover of oxidants from HQ+BSO treatment by not only maintaining basal levels of 8-epi-PGF_{2α}, as these were unchanged by pro-oxidant treatment, but by even enhancing endothelial function. These results suggest ROS upregulation of agonist-stimulated endothelium-dependent vasodilation in the lean Zucker rat. However, the ROS upregulation in the obese Zucker rat was too much for the antioxidant defences to cope with resulting in endothelial impairment.

Clearly a major difficulty in diabetes research is using suitable systems which model disease. However, the pro-oxidant treated obese Zucker rat exhibits a number of

characteristic features of type 2 diabetes, such as hyperglycaemia and glycosuria (data not shown as glucose urine sticks were used – Diabur-test[®] 5000, Roche, UK). This suggests a causative link between oxidant stress and type 2 diabetes, at least in this model. This was unlikely to be related to metabolic deterioration (insulin hyposecretion) due to HQ+BSO toxicity at the level of the endocrine pancreas as the toxicological markers of renal, muscle, exocrine pancreas, and liver damage were previously assessed in our lab and shown to be normal (Vetlab, Horsham, UK). Pathological morphology of the endocrine pancreas was also normal. Decreased insulin levels in plasma can be used as an indicator of pancreatic damage, but this was actually increased in both the pro-oxidant treated lean and obese fasting insulin samples and in the obese glucose stimulated insulin samples (Fig. 6.7A; 6.8A). Interestingly, it is known that insulin resistance also correlates with hyperleptinaemia, a possible candidate to link oxidative stress and endothelial dysfunction in type 2 diabetes.

Oxidant insult due to vascular $O_2^{\cdot-}$ and other ROS generation mechanisms, NO inactivation, increased turnover of antioxidants and the induction of antioxidant enzymes may play a pivotal role in aggravation of metabolic dysfunction. Specifically, the fasting glycaemia and exaggerated fasting hyperinsulinaemia observed in these animals. Increasing oxidant stress and the resulting enhancement then fall of endothelial function (a ‘bell shaped’ response; Fig. 6.11) seems to prevail throughout these experiments, indicating the strong compensatory mechanisms involved in maintaining blood pressure and flow in response to diet. Figure 6.11 shows that increasing amounts of oxidants initially cause endothelial hyperfunction, probably by a compensatory increase in NO production, or alternatively by $O_2^{\cdot-}$ -induced enhancement of cellular responses. Finally, when the level of oxidant stress is increased further, the endothelial hyperfunction starts to diminish and endothelial function measurements are seen as “normal” and comparable to the lean littermate prior to the frank

dysfunction. Overall, assessment of the role of oxidant status on endothelial function and its contribution to metabolic dysfunction in type 2 diabetes needs further investigation.

In summary, there is evidence to link insulin resistance, oxidant stress and altered endothelial function in the obese Zucker rat, a model of pre-diabetes. Oxidant stress alters normal endothelial function by reducing vasodilation and increasing vasoconstriction of the vasculature. This in turn can impair glucose disposal. The obese Zucker rat compensates for this vascular imbalance by enhanced agonist-induced NO-dependent vasodilation. However, by increasing oxidant stress, the obese Zucker rat is unable to cope and modulated endothelial function results. The involvement of other environmental factors, such as diet, in mediating the vascular and metabolic pathology of the Zucker rat is important in furthering the understanding of this disease process.

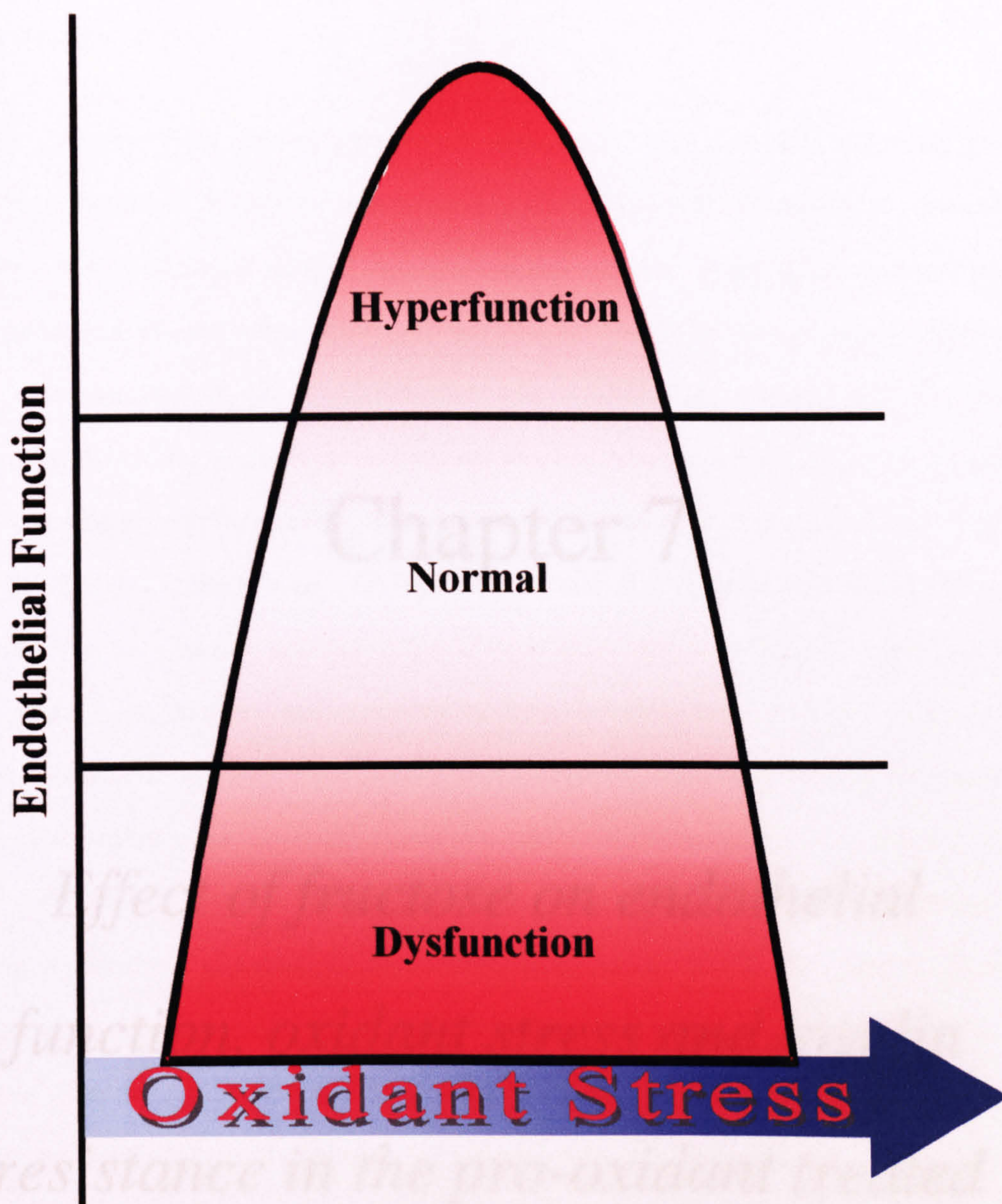


Figure 6.11 Hypothetical representation of changes in endothelial function in relation to the oxidant stress status of the obese Zucker rat.

Chapter 7

*Effect of fructose on endothelial
function, oxidant stress and insulin
resistance in the pro-oxidant treated
obese Zucker rat*

7.1 INTRODUCTION

Previous chapters have shown that hyper endothelial function and impaired glucose tolerance in the obese Zucker rat was worsened by increased ROS-induced by pro-oxidant treatment. It was thought that, if the pro-oxidant-induced endothelial dysfunction and insulin resistance evoked in the obese rats was due to diminished antioxidant defences, then further environmental insult should worsen the dysfunction. Among potential dietary environmental factors, fructose is mainly of interest because of its ability to bypass the control steps of glycolysis, leading to an uncontrolled influx into glycolysis that results in metabolic changes characteristic of type 2 diabetes (i.e. insulin resistance). It is also interesting that the fructose content in our diets is becoming increasingly higher. Over the past 20 years high fructose corn syrup has become a widely used sweetener, especially in non-diet soft drinks. It is not understood if this increasing use of fructose in the population has any relationship to the rising prevalence of type 2 diabetes. Numerous studies in normal rats have shown that fructose feeding can cause hyperinsulinaemia, insulin resistance, and hypertriglyceridaemia (Zavaoni *et al.*, 1980; Tobey *et al.*, 1982). In addition, increased blood pressure (Hwang *et al.*, 1987) and, more recently, defective endothelium-dependent relaxation (Verma *et al.*, 1996) has been shown in this model. Therefore, if a normal rat can be made to develop type 2 diabetes it would follow that a genetically obese and pro-oxidant-induced insulin resistant rat would be even more susceptible to additional deterioration and frank type 2 diabetes.

There is convincing evidence that hyperglycaemia significantly promotes the development of diabetic complications such as retinopathy and neuropathy. High plasma glucose levels may contribute more to this major damage associated with diabetic complications when metabolised to fructose, as glucose metabolised via the polyol pathway results in the formation of intracellular fructose. Fructose is known to accelerate the production of AGE

products more than glucose. However, in hyperglycaemic conditions, glucose is first converted to sorbitol via aldose reductase, then from sorbitol to fructose via sorbitol dehydrogenase. Even with high levels of fructose in diet, the blood level of fructose is only 10 % of that of glucose. However, since the glycation index is 10 times that of glucose, dietary fructose may significantly add to normal glycation that occurs with aging. Fructose also enhances carbonyl stress through the formation of dicarbonyl compounds such as 3-deoxyglucosone (3DG), an intermediate in AGE formation (Thornalley *et al.*, 1984; Sakai *et al.*, 2002).

Metabolism of fructose differs in muscle and liver (Fig. 7.1). Muscle contains only hexokinase, which can phosphorylate fructose to Fructose-6-Phosphate, a direct glycolytic intermediate. The liver contains mostly glucokinase, a glucose specific enzyme, in addition to other enzymes required to utilize fructose for glycolysis. Hepatic fructose is first phosphorylated by fructokinase, yielding fructose-1-phosphate. Aldolase can then utilise both fructose-1,6-biphosphate and fructose-1-phosphate as substrates, to generate dihydroxyacetone phosphate (DHAP) and glyceraldehydes. The DHAP is converted by triose phosphate isomerase to glycerol-3-phosphate that enters glycolysis. This circumvents phosphofructokinase controls, so fructose can thus flood the TCA cycle causing increased lipid synthesis in the liver. Fortunately, humans cannot absorb sufficient fructose to cause a fatty liver; also the metabolism of fructose from the diet is too rapid to allow it to accumulate in the blood. Fructose can cause marginal increases in blood lipids, uric acid, blood insulin, and blood glucose, and worsens already impaired glucose tolerance.

Some of these metabolic effects of fructose are attributed to its rapid hepatic uptake and the fact that it bypasses the phosphofructokinase regulatory step in glycolysis (Mayes, 1993) and this unregulated glycolysis will lead to increased fat synthesis. Acute overloading of the liver with fructose results in sequestration of inorganic phosphate in fructose-1-

phosphate. The metabolism of fructose results in increased xanthine oxidase activity and glyceraldehyde production (which can generate free radicals). It may be that the liver is exposed to oxidative stress following fructose consumption and this may be a possible mechanism to partly explain the induced liver abnormalities.

The fructose fed rat is a well established convenient, non-obese *in vivo* model of insulin resistance and type 2 diabetes (Tobey *et al.*, 1992; Faure *et al.*, 1997). Consequently, it can be seen to simulate what happens in humans, i.e. a disorder created by feeding abnormalities. Rats fed fructose either in the drinking water or the chow for a number of weeks (usually between 2-6 weeks) exhibit characteristic hyperglycaemia, insulin resistance and impaired endothelial function (Prasad *et al.*, 1998). Additionally, this model has increased triglycerides plasma, and significantly raised levels of malondialdehyde and glycation products, indicating oxidative stress and glycation stress (Faure *et al.*, 1997). The mechanism underlying endothelial function and its relationship to oxidant stress in these rats is not however understood. Since we have good evidence to associate oxidative stress, insulin resistance and endothelial function in the pro-oxidant treated obese Zucker rat, we wished to further examine our hypothesis in another established model of diabetes, i.e. the fructose fed rat. We reasoned this to be an important additional measure in the validation of oxidative stress hypothesis.

7.2 RESULTS

7.2.1 Effect of fructose and pro-oxidant treatment on insulin resistance in the obese Zucker rat.

Body weight was greater in obese relative to lean animals ($P<0.05$) (table 7.1). The fructose only lean and obese groups showed only a slight (30-50 g) increase in weight compared to control and pro-oxidant treatments ($P>0.05$).

Treatment	Control	Fructose	HQ+BSO	Fructose & HQ+BSO
Obese	406.4± 8.6*	451.7±10.2*	399.3±19.5*	380.0±18.7*
Lean	278.8±7.3	314.3±10.3	303.6±7.7	301.4±6.9

Table 7.1 Body weights (g) of lean and obese Zucker rats following a fructose diet and pro-oxidant administration. Data are expressed as mean±s.e.mean of 5-7 determinations. * $P<0.05$ with respect to corresponding treatment in the lean group.

Plasma insulin and glucose levels were altered by pro-oxidant treatment and fructose diet. Obese basal plasma glucose and insulin levels were elevated relative to lean levels ($P<0.05$) in all groups with the exception of the fructose fed only group. Here fasting glucose levels were similar in the lean and obese groups (Fig. 7.2). Fructose treatment elevated basal plasma insulin levels in lean ($P<0.05$) but not obese rats ($P>0.05$). Similarly, basal glucose levels were significantly increased by HQ+BSO treatment but not HQ+BSO in combination with fructose, in lean but not obese rats (Fig. 7.2).

Following bolus glucose (0.5 g kg⁻¹ i.v.) administration, both plasma glucose and insulin were greater in obese animals (Fig. 7.3) this was similar in all groups. Treatment with HQ+BSO alone caused increased insulin levels in the obese compared to their obese

control group. Comparisons of the insulin/ glucose ratio showed a similar profile to that of insulin and glucose AUC, i.e. impairment in glucose disposal in obese compared to lean rats and no significant changes with pro-oxidant treatment in obese and lean groups (Fig. 7.3C).

7.2.2 Effect of fructose and pro-oxidant treatment on endothelial function in the obese Zucker rat.

Comparison of *pD*₂ values from ACh-induced vasorelaxation showed a greater relaxation in the obese aortic rings compared to lean (*P*<0.05). Vasorelaxation was significantly impaired in the obese treated with HQ+BSO treatment alone, and in combination with fructose (*P*<0.05), but not by fructose alone (*P*<0.05) (Fig. 7.4; Table 7.2).

	Treatment			
<i>E</i> _{max} (%)	Control	Fructose	HQ+BSO	Fructose & HQ+BSO
Obese	95.8±1.8	94.7±1.6	91.7±2.3	93.1±0.9
Lean	90.1±2.0	86.6±2.8	88.1±2.7	90.5±1.5

<i>pD</i> ₂	Control	Fructose	HQ+BSO	Fructose & HQ+BSO
Obese	7.50±0.03*	7.45±0.03*	7.16±0.03 ^Ψ	7.25±0.02 ^Ψ
Lean	7.18±0.03	7.26±0.01 ^Ψ	7.16±0.01	7.28±0.01 ^Ψ

Table 7.2 Effects of combined fructose and pro-oxidant treatment on vasodilation to ACh (0.0001-10 nM) in aortic rings of obese and lean Zucker rats. Data are expressed as mean±s.e.mean of 4-7 determinations. **P*<0.05 with respect to corresponding lean group; ^Ψ*P*=0.05 with respect to lean or obese control group.

In contrast, the lean vasorelaxation was enhanced by fructose treatment, even in combination with HQ+BSO. Interestingly, the obese aortic rings were only comparable to the lean rings following pro-oxidant treatment, with or without fructose. However, when E_{\max} values were compared, there was no difference between groups. Vasorelaxations in the presence of L-NAME were significantly and maximally inhibited in all groups.

	Treatment			
E_{\max} (g)	Control	Fructose	HQ+BSO	Fructose & HQ+BSO
Obese	2.40±0.18 [†]	2.47±0.07 [†]	2.45±0.24 [†]	2.66±0.13 [†]
Lean	2.65±0.16 [†]	2.74±0.22 [†]	2.77±0.14 [†]	2.61±0.08 [†]

pD_2	Control	Fructose	HQ+BSO	Fructose & HQ+BSO
Obese	7.00±0.06 ^{*†}	6.79±0.04 [†]	6.76±0.06 ^{ψ†}	6.85±0.06 [†]
Lean	6.74±0.04 [†]	6.82±0.05 [†]	6.89±0.04 [†]	6.97±0.06 ^{ψ†}

Table 7.3 Effects of combined fructose and pro-oxidant treatment on vasocontraction to Phe (0.0001-10 nM) in aortic rings of obese and lean Zucker rats. Data are expressed as mean±s.e.mean of 5-7 determinations. * $P<0.05$ with respect to corresponding lean group; $^{\psi}P<0.05$ with respect to the equivalent lean or obese control; [†] $P<0.05$ with respect to the equivalent L-NAME treated lean or obese control.

Comparison of Phe elicited concentration responses using pD_2 values showed a leftward shift in the contraction response of the obese relative to lean ($P<0.05$) (Fig. 7.4; table 7.3). However, this was not mirrored by an increase in E_{\max} , which was actually smaller in the obese ($P>0.05$). The presence L-NAME caused a leftward shift in both lean and obese contraction curves, normalising these differences between lean and obese Zucker rats ($P<0.05$) (Fig. 7.4; table 7.4). Treatments with HQ+BSO alone caused significant increases

in Phe-induced contractions in obese but not lean rats. However, the lean rats showed increased contractions with fructose & HQ+BSO combined treatment ($P<0.05$). The presence of L-NAME again evoked a leftward shift in all treatments groups ($P<0.05$), resulting in comparable contractions. Additionally, comparison of the E_{\max} values showed no differences between treatment groups in lean or obese rat aortic rings. In the presence of L-NAME, all groups had increased E_{\max} values ($P<0.05$), but were all comparable within the pro-oxidant treatment groups (Fig. 7.4; table 7.4).

	Treatment			
E_{\max} (g)	Control	Fructose	HQ+BSO	Fructose & HQ+BSO
Obese	3.8±0.1	3.9±0.2	3.8±0.2	3.9±0.2
Lean	3.8±0.2	4.0±0.2	4.0±0.1	3.9±0.2

pD_2	Control	Fructose	HQ+BSO	Fructose & HQ+BSO
Obese	7.54±0.10	7.51±0.08	7.48±0.09	7.58±0.11
Lean	7.55±0.10	7.52±0.09	7.58±0.10	7.36±0.04

Table 7.4 Effects of combined fructose and pro-oxidant treatment on vasocontraction to Phe (0.0001-10 nM) in the presence of L-NAME (300 µM) in aortic rings of obese and lean Zucker rats. Data are expressed as mean±s.e.mean of 6-7 determinations.

Comparison of SNP-induced vasorelaxation using E_{\max} values showed similar results in the lean and obese rat aortic rings. This vasorelaxation was blunted by fructose in the lean groups ($P<0.05$) (Table 7.5). All other treatments group showed no changes from control.

	Treatment			
E_{\max} (%)	Control	Fructose	HQ+BSO	Fructose & HQ+BSO
Obese	100.2±0.6	99.9±1.0	99.9±1.0	100.8±0.8
Lean	100.2±0.7	97.1±1.1 ^ψ	98.7±0.6	100.1±0.5

E_{\max} (%) in the presence of L-NAME	Control	Fructose	HQ+BSO	Fructose & HQ+BSO
Obese	100.7±1.8	98.3±0.6	96.7±1.3	97.5±0.8
Lean	97.7±1.2	97.5±0.9	96.5±0.7	96.0±0.9

Table 7.5 Effects of combined fructose and pro-oxidant treatment on vasodilation to SNP (100 nM) in aortic rings of obese and lean Zucker rats. Data are expressed as mean±s.e.mean of 6-7 determinations. ^ψ*P*<0.05 with respect to the lean control.

Enhanced vasodepressor activity to ACh and SNP following fructose and HQ+BSO combination treatment in obese Zucker rats in vivo. Vasodepression to bolus dose ACh (0.01- 10 nmol kg⁻¹ i.v.) was comparable in lean and obese rats on control diet, but became greater in the obese relative to the lean rats after fructose and HQ+BSO combination treatment (*P*<0.05 2W ANOVA) (Fig. 7.5). Similarly, vasodepression to bolus dose SNP (0.1- 100 nmol kg⁻¹ i.v.) was comparable in the lean and obese rats on control diets, and was not significantly greater in the obese relative to the lean following treatment.

High mean arterial blood pressure in obese Zucker rats. Mean arterial blood pressure in the obese control and treatment groups was higher than that of the lean (*P*<0.05) Zucker rat groups (Fig. 7.6). Pro-oxidant and fructose treatment showed no effect on the blood pressure in either group.

High plasma nitrate/nitrite levels in obese Zucker rats. Plasma nitrate/nitrite levels in the obese control and treatment groups were higher than those of the lean ($P<0.05$) Zucker rat groups (Fig. 7.7). In the obese rats, fructose and HQ+BSO treatments showed only a slight increase in plasma nitrate/nitrite levels compared to the controls and fructose in combination with HQ+BSO ($P>0.05$). In the lean Zucker rat groups HQ+BSO was reduced compared to the lean untreated ($P<0.05$).

7.2.1 Effect of fructose and pro-oxidant treatment on oxidant stress in the obese Zucker rat.

Plasma levels of 8-epi-PGF_{2α} were only slightly increased in the obese relative to the lean Zucker rats, an increased n number may have attained significance (Fig. 7.8). Treatment with HQ+BSO, but not fructose or fructose combined with HQ+BSO, increased plasma 8-epi-PGF_{2α} levels in obese ($P<0.05$). Dietary antioxidant treatments showed no effect in the lean ($P>0.05$) Zucker rats.

High plasma triglyceride levels in obese Zucker rats. Plasma triglyceride levels in the obese control and treatment groups were higher than that of the lean ($P<0.05$) Zucker rat groups (Fig. 7.9).

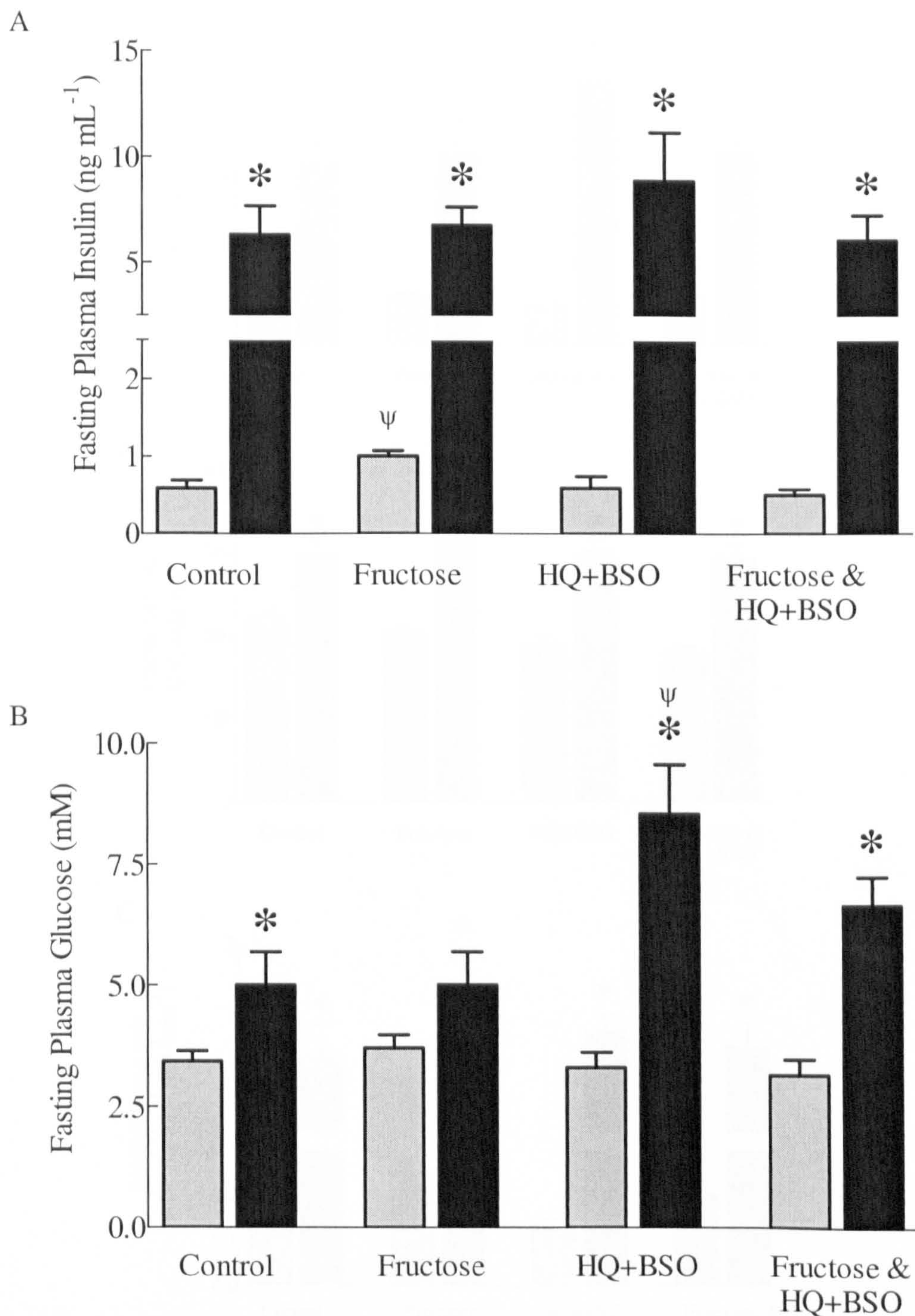


Figure 7.2 Measurement of basal plasma insulin (ng mL⁻¹) and glucose (mM) in 13 week-old lean (■) and obese (■) Zucker rats treated with sham, fructose, and HQ+BSO alone and in combination. Data are expressed as mean±s.e.mean of 5-7 determinations. Statistical significance was compared using a student t-test, * $P < 0.05$ with respect to the equivalent lean control, or using one way ANOVA, ^Ψ $P < 0.05$ with respect to the control treatment group.

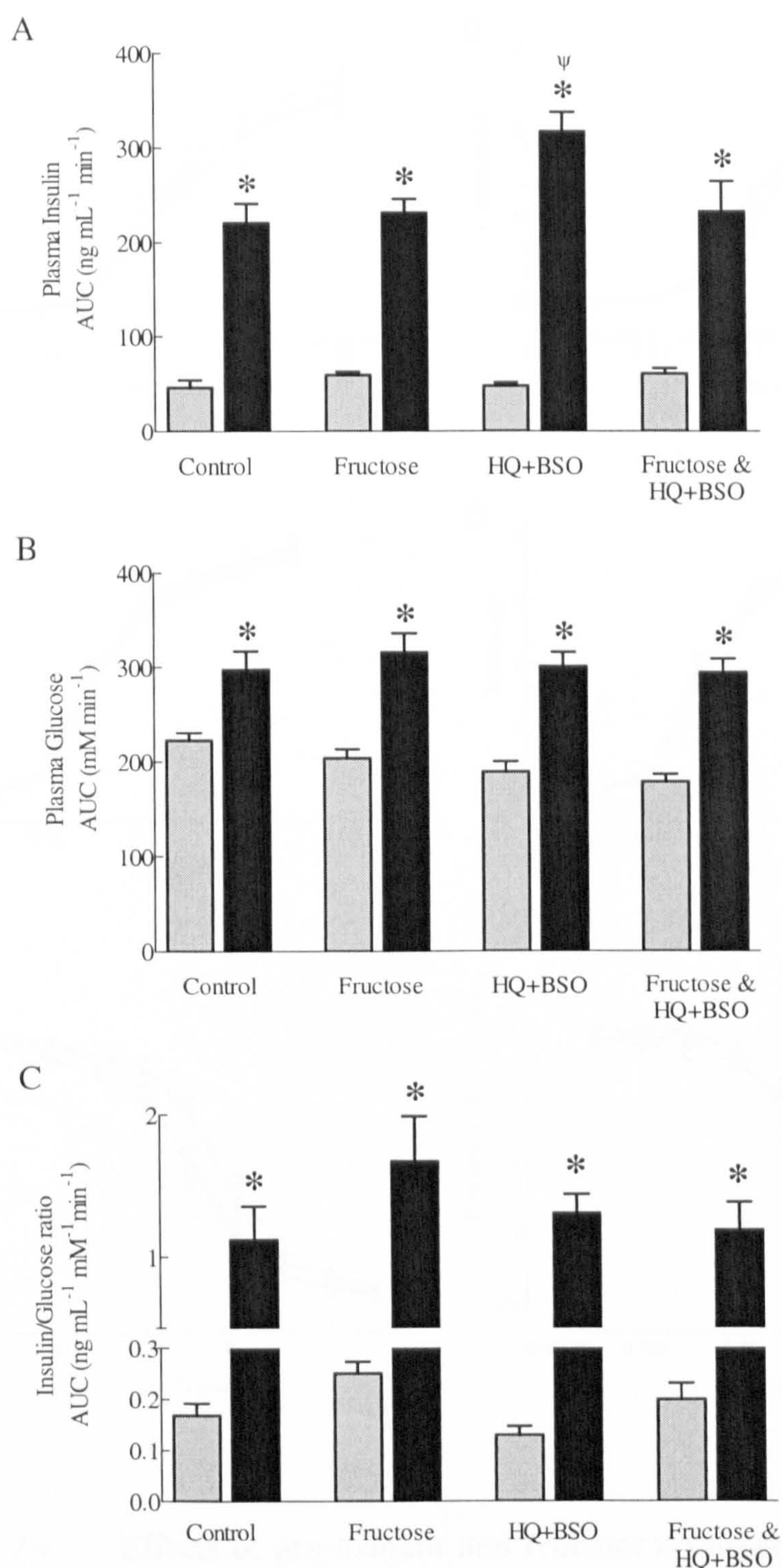


Figure 7.3 Glucose tolerance test (g.t.t) in 13 week-old lean and obese **Zucker rats** treated with sham, fructose, hydroquinone and buthionine sulfoxamine (HQ+BSO) (50 mg kg⁻¹ i.p. daily), and fructose (HQ+BSO) in combination. Data are expressed as mean±s.e.mean of 4-7 determinations. Statistical significance was compared using a student t-test, **P*<0.05 with respect to the equivalent lean control, or using one way ANOVA, ^Ψ*P*<0.05 with respect to the control treatment group.

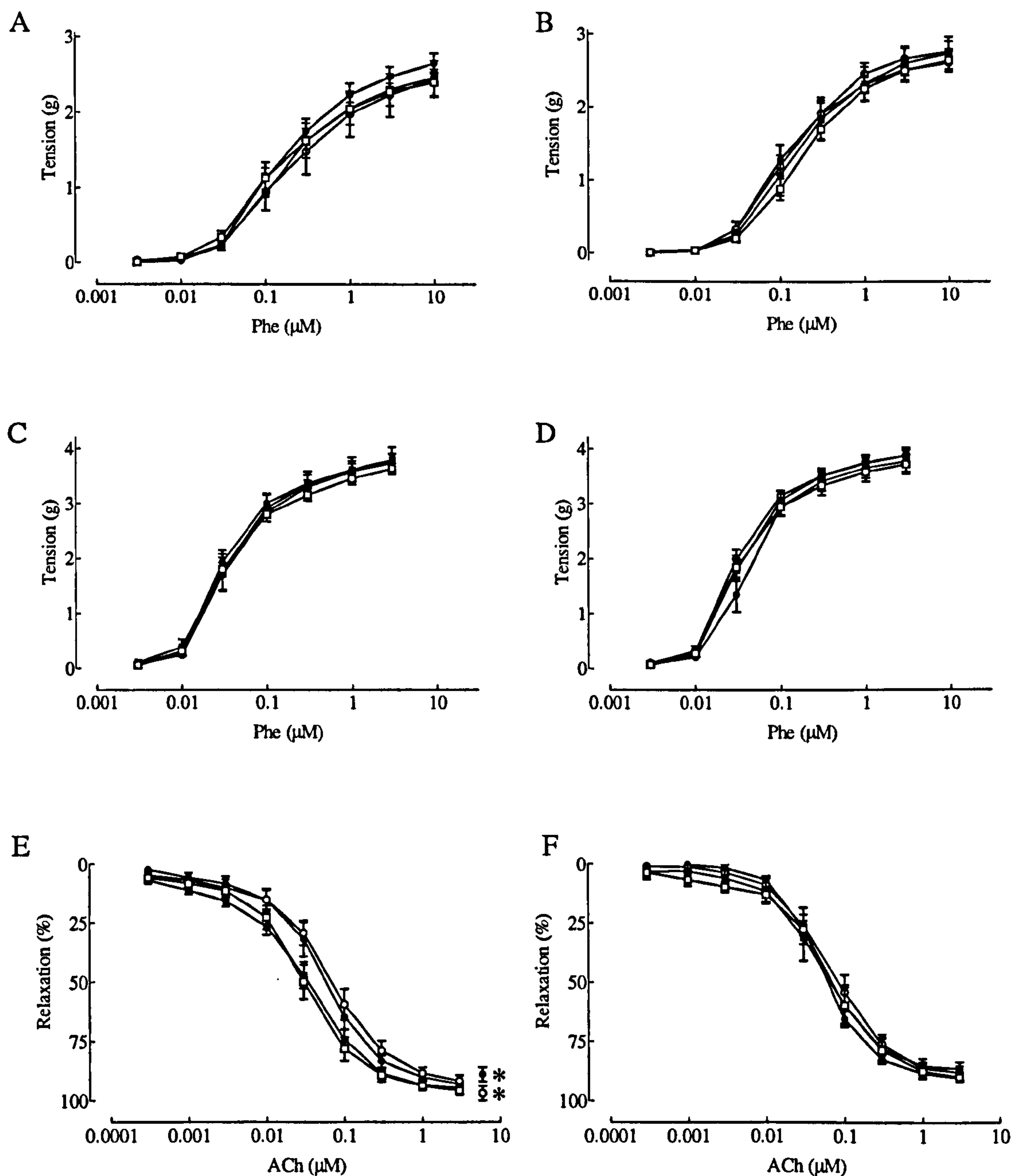


Figure 7.4 Effects of pro-oxidant and fructose treatment on contraction and relaxation responses in aortic rings in 13 week-old lean and obese Zucker rats. Rats were treated with sham (□), fructose (■), hydroquinone and buthionine sulfoxamine (HQ+BSO) (○), and fructose (HQ+BSO) in combination (●). Contraction responses to phenylephrine (0.03– 10 μM) alone in obese (A) and lean (B) rats, and in the presence of L-NAME (300 μM) in obese (C) and lean (D) rats. Vasorelaxation responses to acetylcholine (ACh, 0.0003–3 μM) in obese (E) and lean (F) rats. Data are expressed as mean \pm s.e. mean of 4-7 determinations. Statistical significance (*) using 2 way ANOVA was considered established at $P < 0.05$.

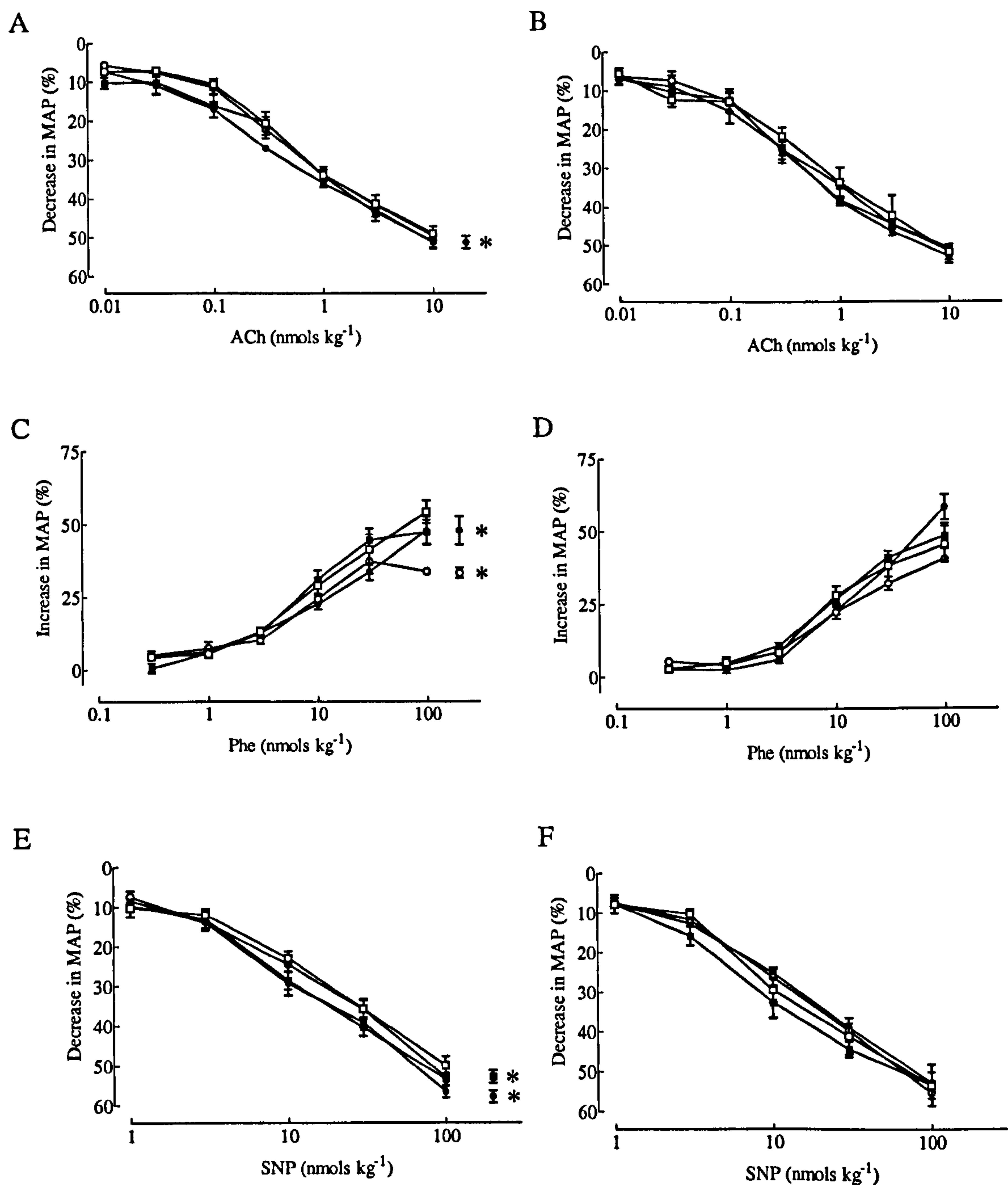


Figure 7.5 Effects of pro-oxidant and fructose treatment on vasodilator and vasoconstrictor responses in 13 week-old lean and obese Zucker rats *in vivo*. Rats were treated with sham (□), fructose (■), hydroquinone and buthionine sulfoxamine (HQ+BSO) (○), and fructose (HQ+BSO) in combination (●). Graphs show vasodilator responses to acetylcholine (ACh, 0.001-10 nmol kg⁻¹) in obese (A) and lean (B) rats, and sodium nitroprusside (SNP 1-100 nmol kg⁻¹) in obese (E) and lean (F) rats and vasoconstrictor responses to phenylephrine (0.1– 100 nmol kg⁻¹) in obese (C) and lean (D) Zucker rats. Data are expressed as mean±s.e.mean of 4-7 determinations. Statistical significance (*) using 2 way ANOVA was considered established at $P<0.05$.

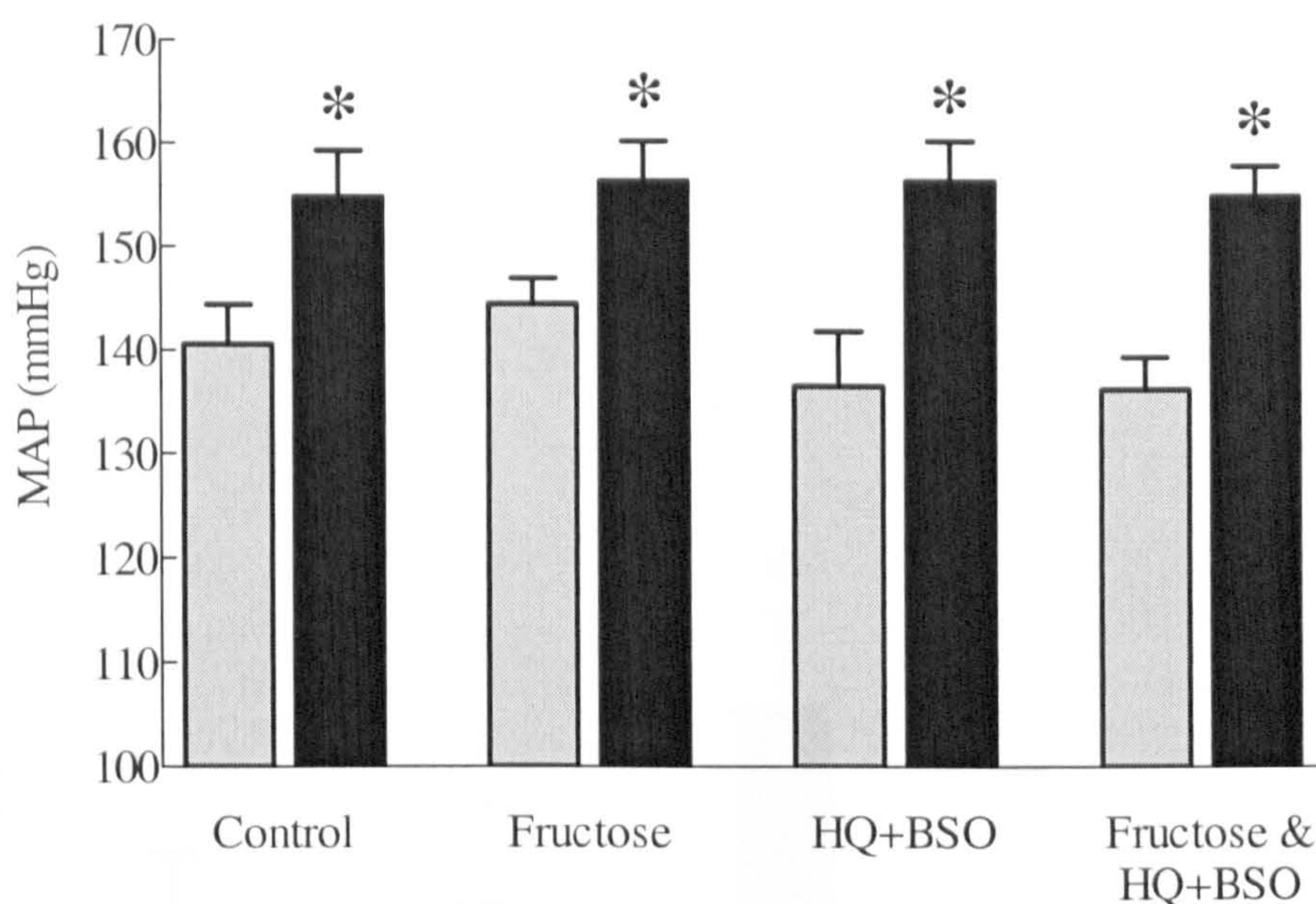


Figure 7.6 Mean arterial blood pressure (MAP) in anaesthetised 13 week-old lean (■) and obese (■) Zucker rats treated with sham (□), fructose, hydroquinone and buthionine sulfoxamine (HQ+BSO), and fructose (HQ+BSO) in combination. Data are expressed as mean±s.e.mean of 6-7 determinations. Statistical significance was compared using a student t-test, * $P < 0.05$ with respect to the equivalent lean control group.

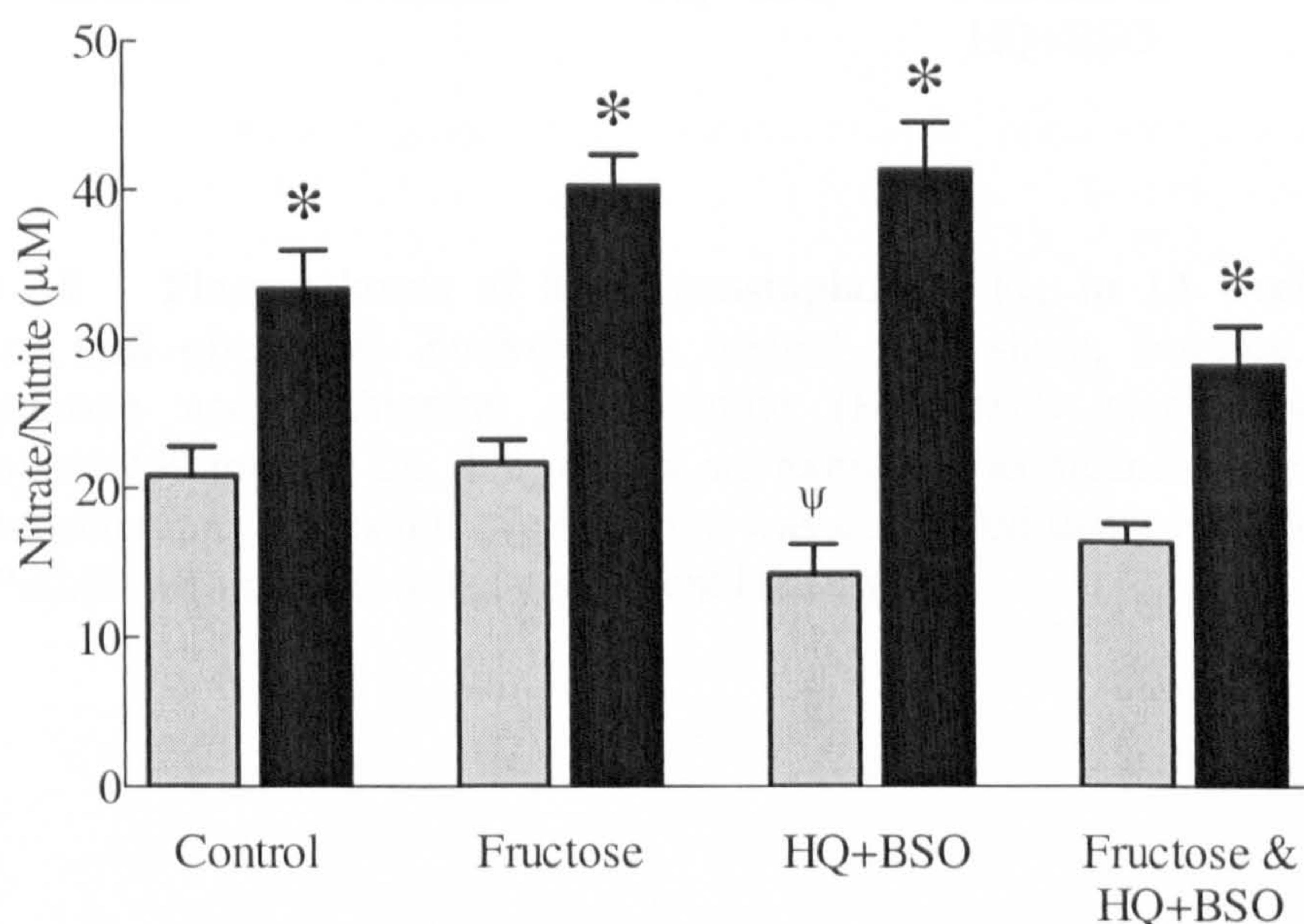


Figure 7.7 Plasma nitrate/nitrite levels in lean and obese 13 week-old Zucker rats treated with sham, sham, fructose, and hydroquinone and buthionine sulfoxamine (HQ+BSO) alone and in combination (50 mg kg⁻¹ i.p. daily). Data are expressed as mean±s.e.mean of 3-6 determinations. Statistical significance was compared using a student t-test, * $P < 0.05$ with respect to the equivalent lean control, or using one way ANOVA, ^Ψ $P < 0.05$ with respect to the control treatment group.

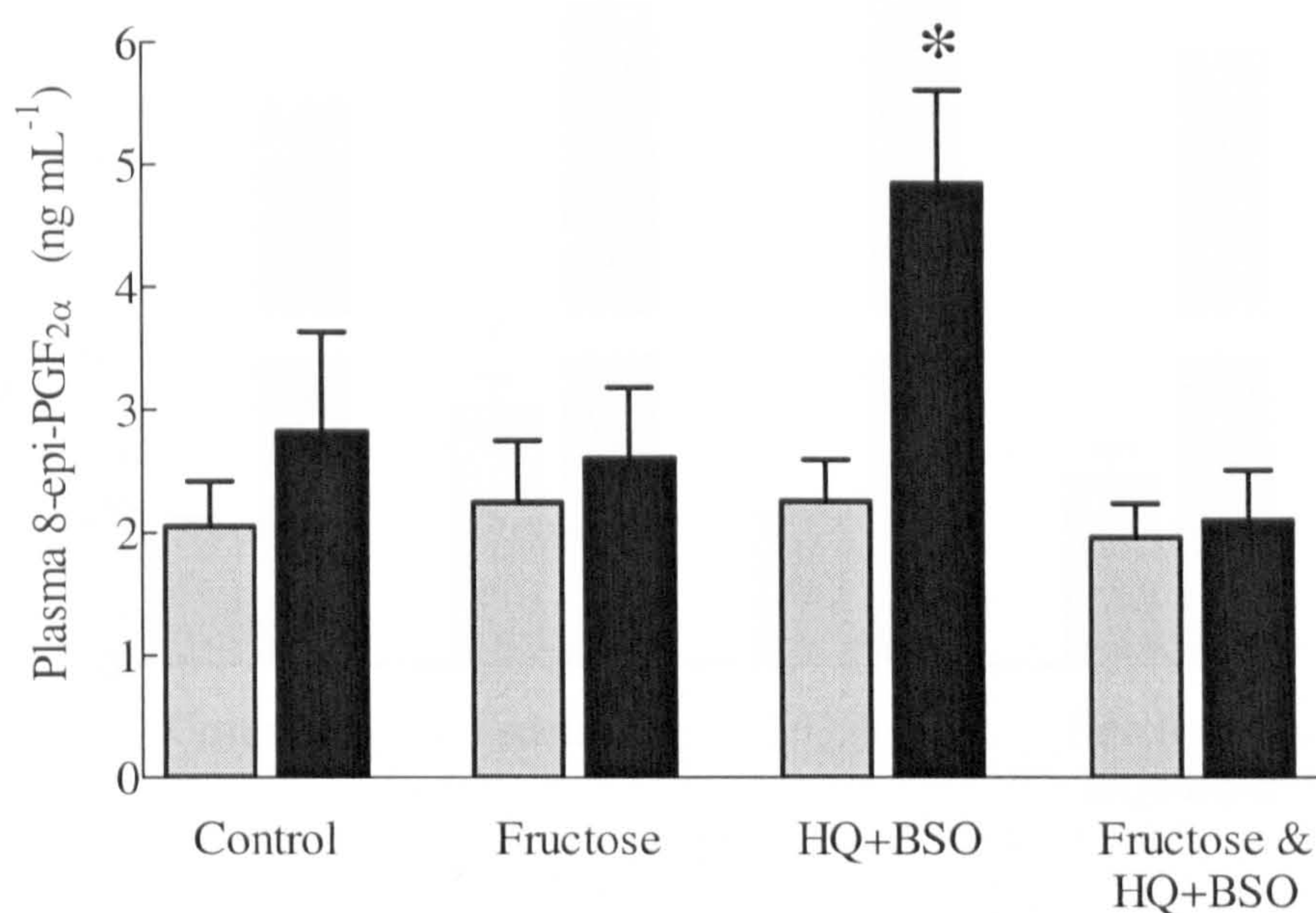


Figure 7.8 Plasma levels of 8-epi prostaglandin $F_{2\alpha}$ in 13 week-old lean (□) and obese (■) Zucker rats treated with sham, fructose, and hydroquinone and buthionine sulfoxamine (HQ+BSO) alone and in combination (50 mg kg⁻¹ i.p. daily). Data are expressed as mean±s.e.mean of 4-7 determinations. Statistical significance was compared using a student t-test, * $P<0.05$ with respect to the equivalent lean control.

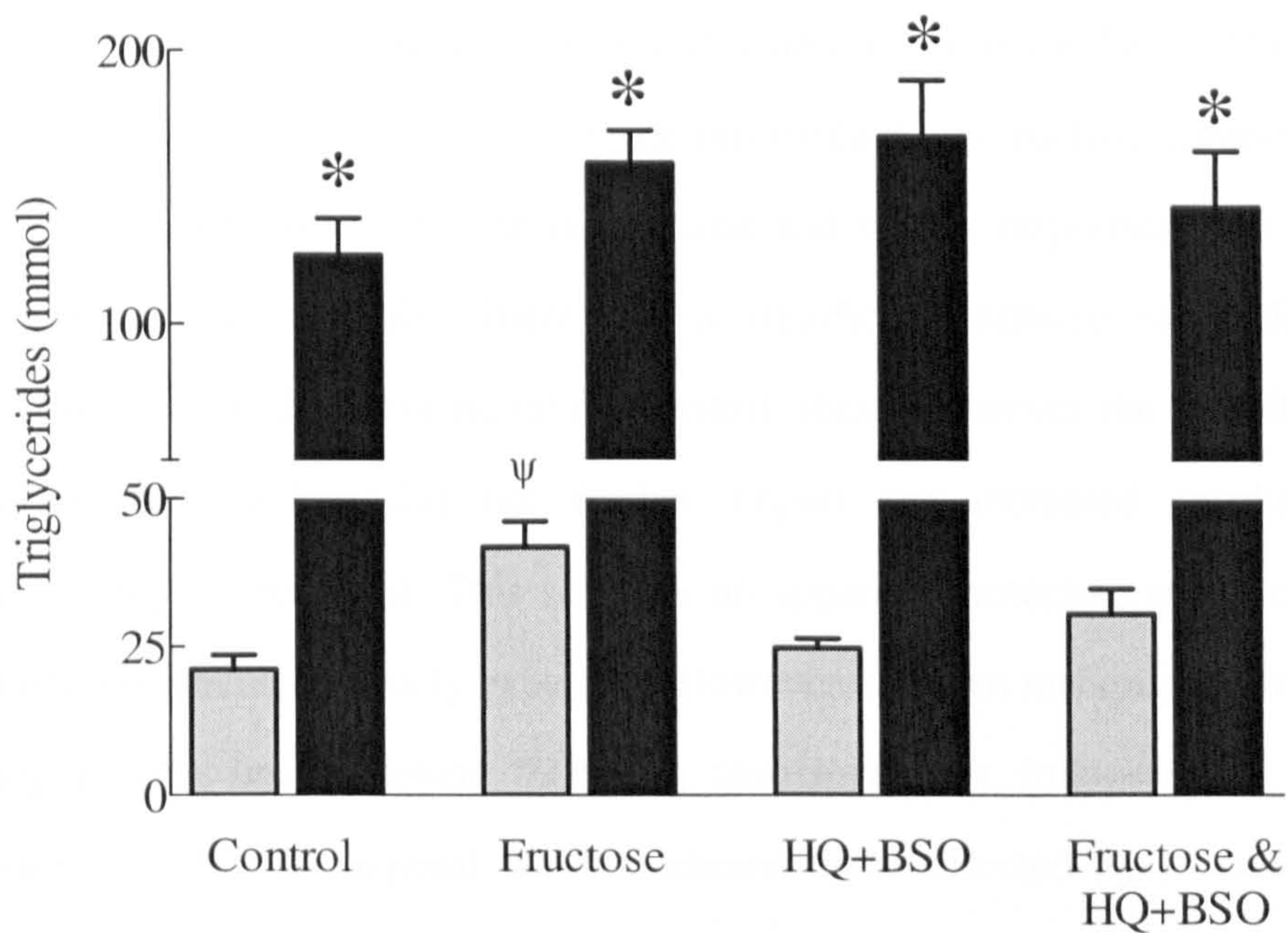


Figure 7.9 Fasting plasma triglyceride levels in lean and obese 13 week-old Zucker rats treated with sham, fructose, and hydroquinone and buthionine sulfoxamine (HQ+BSO) alone and in combination (50 mg kg⁻¹ i.p. daily). Data are expressed as mean±s.e.mean of 5-7 determinations. Statistical significance was compared using a student t-test, **P*<0.05 with respect to the equivalent lean control, or using one way ANOVA, ^Ψ*P*<0.05 with respect to the control treatment group.

7.3 DISCUSSION

Obese basal plasma glucose and insulin levels were significantly elevated relative to lean levels. Interestingly, plasma glucose was increased in the obese HQ+BSO treatment group, a condition that was normalized with the pro-oxidant and fructose combination. Similarly, following the glucose load, both the glucose and insulin responses were greater in obese compared to lean animals. There was a significant increase in insulin levels in the HQ+BSO treated obese compared the control obese. However the HQ+BSO combination with fructose feeding did not further impair the increased insulin response, but paradoxically decreased it. This suggests an apparent protective effect of fructose rather than impairment as originally expected. However, the lean rats only showed an increase in fasting plasma insulin levels following treatment with fructose only. Fasting plasma glucose and glucose disposal was not shown to be affected by fructose or pro-oxidant treatment in the lean rats. Pro-oxidant treatment was not expected to increase the insulin resistance parameters tested in this study as previous studies (chapter 6) have not shown impairment in the lean rats. This was thought to be due to their ability to compensate for this increased stress.

Comparison of ACh-induced vasorelaxation was significantly greater in the obese aortic rings compared to lean. This finding has been shown by other groups (Sexl *et al.*, 1985) and supports the enhanced vasodilation found in the obese hindquarters. The obese vasorelaxation was significantly impaired by HQ+BSO treatment alone. However, this was not the case with HQ+BSO in combination with fructose. This again suggests that fructose may indeed protect against pro-oxidant damage, at least in this model. All these responses were in similarly contracted rings, as the Phe elicited contraction curves were not different between the treatment groups of either the obese or lean rats. However, the Phe contractions were increased in the obese aortic rings compared to the lean using pD_2 value

comparisons. Though if this increased contraction was to affect the ACh-induced relaxations it would cause impairment, as the more contracted a tissue is, the higher the concentration of vasodilatory agonist required to overcome the contraction. The SNP-induced vasorelaxations were similar in the lean and obese rat aortic rings, and therefore any changes measured in the obese are endothelial-dependent.

Blood pressure studies showed vasodepressor activity to ACh and SNP was enhanced following fructose and HQ+BSO combination treatment in obese Zucker rats *in vivo*. Vasodepression to bolus dose ACh and SNP was comparable in lean and obese rats on control diet, but became greater in the obese relative to the lean rats after fructose and HQ+BSO combination treatment. This data suggests a protective role for fructose in pro-oxidant treated rats. Furthermore, as mean arterial blood pressure was unaffected by treatments in either lean or obese groups, agonist-induced changes could not simply be greater due to higher basal pressures. The obese control and treatment groups did have significantly higher blood pressures than those of the lean Zucker rat groups. However, this did not cause an increased agonist-induced response in the obese.

Interestingly, plasma nitrate/nitrite levels in the obese control and treatment groups were higher than those of the lean Zucker rat groups. This was not unexpected given the increased ACh-induced endothelial-dependent vasodilation seen in the obese rats. However, in these rats, fructose and HQ+BSO treatments showed a only slight increase in plasma nitrate/nitrite levels compared to untreated and those treated with fructose and HQ+BSO in combination. These results support findings from previous insulin resistance and endothelial-dependent studies. In addition, the lean pro-oxidant treated group showed decreased levels of nitrate/nitrite, suggesting that the NO balance is affected by pro-oxidant treatment, but not enough to impair endothelial function. Finally, oxidant stress measurements showed treatment with HQ+BSO, but not fructose or fructose and HQ+BSO

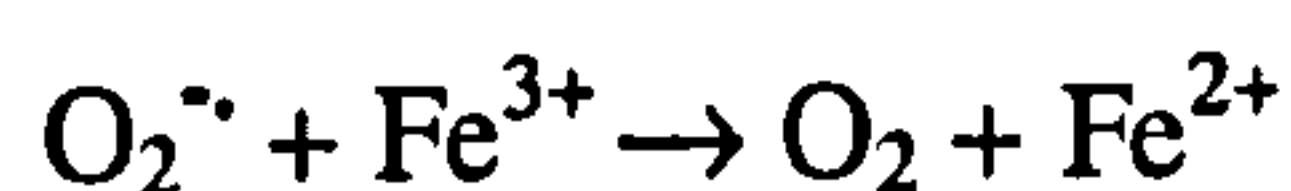
in combination, increased plasma 8-epi-PGF_{2α} levels in the obese rats. The lean plasma 8-epi-PGF_{2α} levels remained unaffected by pro-oxidant treatment; this has been seen in previous experiments (chapter 6). The plasma 8-epi-PGF_{2α} levels in this study were analysed using an ELISA method unlike the previous chapters where GCMS was used. It is interesting to note that similar levels were obtained in this study compared to the previous findings.

The protective role of fructose shown in these studies needs further investigation and this data needs to be confirmed; particularly as the lean Zucker rat only responded to the fructose diet with increased fasting insulin and triglyceride levels, and no changes in endothelial function or oxidant stress were shown. Changes in endothelial function and oxidant stress have previously been shown in Wistar rats following a fructose treatment (Dai & McNeill, 1995; Faure *et al.*, 1997). In addition, studies in the Zucker rat have shown increased insulin and triglycerides as well as increased blood pressure in fructose fed lean Zucker rats (Verma *et al.*, 2001). Interestingly, this study also showed that fructose increased insulin levels in the obese Zucker rat but that there was no effect on blood pressure. These results are supportive of my data in that a fructose diet in the obese rats was shown to have a protective effect on aortic ring vasodilation and no effect on blood pressure. Unfortunately, in my studies the obese rats did not develop a worsened hyperinsulinaemia. However, another study in the obese Zucker rats that supports my data has shown that small amounts of oral fructose reduced post prandial glycaemia (Wolf *et al.*, 2002). It may be that my results did not show fructose alone induced increases in insulin and triglyceride levels in the obese rat because both parameters were already high. Alternatively, it may be that fructose promotes diabetes in the absence of oxidant stress in normal albino animals, but is protective when levels of oxidant stress is high or levels of antioxidants are low.

This data appears to be the first demonstration of fructose feeding protecting against the deterioration of metabolic disease leading to frank diabetes, at least in the model described. It is important to note that previous studies have shown that a fructose diet protects against the complications of diabetes such lethal reperfusion injury to sinusoidal endothelial cells in rat livers (Currin *et al.*, 1996). In addition, high fructose levels have been shown to protect against LPS-induced sepsis damage (Harris *et al.*, 1999). It was speculated that this may be due to the high circulating levels of endogenous triglyceride rich lipoproteins, which are components of a non-adaptive innate immune response to endotoxin-induced by fructose. Interestingly, triglyceride levels were increased in the obese rats in our studies (Fig. 7.9). Also, increased levels were seen in the lean rats with fructose diet alone. In addition, Harris *et al.*, showed increased levels of NO released from endothelial cells. When the induced NO was non-selectively inhibited, the fructose-induced protection against endotoxemia *in vivo* was completely abolished. Similarly, hepatocytes were protected from TNF α -induced toxicity by fructose *in vivo* and *in vitro* (Latta *et al.*, 2000), though ATP depletion by fructose was suggested as the primary metabolic cause of their results. Fructose does not block ATP synthesis nor deplete organic substrates for glycolysis or mitochondrial energy generation. It is thought that fructose acts as a sink for the cellular phosphate pool, as it is rapidly phosphorylated in order for it to enter into glycolysis. Interestingly, a study has shown that by increasing ingestion of fructose in healthy subjects, levels of uric acid increased (MacDonald *et al.*, 1978). This supports the theory that fructose causes the depletion of hepatic adenine nucleotides and that the purine component of these appears as uric acid. Indeed the authors show a significant correlation between serum fructose and uric acid concentrations. For each 1 g fructose /kg bodyweight a 0.6 mg/100 mL increase in uric acid resulted. Uric acid is an antioxidant *in vivo* that scavenges peroxy radicals, HO \cdot and ONOO \cdot . Therefore in my experiments this increased uric acid concentration may have scavenged the basal radical production in the obese Zucker rats and even the pro-oxidant challenge which in turn prevented endothelial dysfunction. Most interestingly, whilst

preventing oxidant-induced endothelial dysfunction, fructose treatment may have also prevented in the obese, the insulin and triglyceride increases seen in the lean rats.

Other explanations for the protection by fructose include intracellular acidification as a result of glycolysis. However, it has been shown that its protection is pH-dependent in the hepatocyte (Nieminen *et al.*, 1990). Another likely theory proposed is the protection against oxidative injury by ferric iron (Fe^{3+}) chelation (Valeri *et al.*, 1997). Fructose and tagatose were shown to protect hepatocytes against oxidative cell injury by the formation of stable complexes with Fe^{3+} . Effective iron chelation would prevent iron catalysed formation of HO^\cdot and prevent pro-oxidant-induced toxicity. For example the Fenton reaction is the reaction involved in the biological generation of HO^\cdot . The reaction involves $\text{O}_2^{\cdot-}$ which reduces Fe^{3+} to Fe^{2+} and then subsequent reduction of H_2O_2 re-oxidizes the Fe^{2+} with the concomitant generation of HO^\cdot :



Interestingly, these studies showed that toxicity from both cocaine and nitrofurantoin was dependent on the presence of intracellular Fe^{3+} , and that deferoxamine protected against injury (Valeri *et al.*, 1997). The authors considered whether Fe^{3+} chelation is the factor in fructose protection, and whether decreased intracellular Fe^{3+} would prevent the rapid oxidation of GSH. This has been shown in reverse in studies where fructose inhibited reoxygenation-induced apoptosis by decreasing ROS via stabilisation of the glutathione pool (Frenzel *et al.*, 2002). These studies showed that pre-treatment with BSO abolished the effects of fructose. There may be a dual role for intracellular Fe^{3+} in fructose protection as it has also been shown that Fe^{3+} is required for uric acid to protect against ONOO^\cdot . In conclusion, our studies show a protective role by fructose to prevent the onset of frank

diabetes. However, it remains clear that a more complete understanding of the cytoprotective effects of fructose is needed.

Chapter 8

General Discussion

The models developed for my research included one for blood flow measurements in the rat ear – a potential model of non invasive endothelial function. This model was particularly interesting as it provided *in vivo* data that supported *in vitro* studies which showed the existence of an EDHF involvement in small resistance vessels (Urakami-Harasawa *et al.*, 1997). However, there were a number of problems with the methodology which restricted the time and effort afforded to this method's development, hence its absence in the later chapters. Firstly, the probe was rather large and bulky in comparison to the small delicate ear of the rat. This impeded the ability to position the tip (only 0.85 mm itself) carefully on the same vessel resulting in large variations in each experiment. Secondly, slight movements from the rat ear, such as a very deep breath, caused the probe position to move. If the probe was smaller and more delicate it could be secured to a sticky backing such as those used in rabbit ear photoplethysmography studies and this would avoid the movement. The results shown have accounted for the movement in the studies by using SNP as an internal control, though a number of rats were not included in the data sets as, due to many hours of anaesthetic and repeated dosing of ACh, they were near to being volume loaded. Future studies in this model could therefore be achieved if tiny probes were used with gentle adhesive and with a small pliable wire that could transmit signals from the probe to the recorder.

EDHF involvement in the resistance vessels is certainly an area of great interest. Preliminary evidence from our laboratory showed a non-NO non-prostanoid relaxation of obese Zucker rat aortic rings (Kaw *et al.*, 1999) indicating that EDHF is an important factor in endothelial dysfunction in the Zucker rat, making this potential non invasive *in vivo* model a valuable tool for investigation of the role of EDHF in diabetes.

The perfusion model was a reproducible and simple preparation. It allowed investigation into the insulin sensitive beds and resistance vessels that are most important in the

investigation of type 2 diabetes. An example of the importance of preparation choice was seen, in that very different results were achieved in the parallel studies of the acute effects of insulin in the rat aortic rings and hindquarters preparation (chapter 5). This shows how the site of investigation is particularly significant in diabetes studies. In hindsight, studies in the fructose chapter should have been done in the hindquarters preparation as the data would have probably provided a greater correlation between endothelial function and oxidant stress. As far as future development of this model is concerned, glucose uptake from the hindquarters might be measured. This measurement would provide another important physiological parameter similar to the euglycaemic clamp technique – the gold standard in glucose disposal measurements. Radioactive tracers of glucose then could be used in the PSS, and glucose content in the muscles and effluent measured following the experiment. Preliminary studies have been carried out and present a promising experimental model for future studies. The limitation of the perfused hindquarters preparation was the time restriction, in that 90 min was used as a maximum time for the experimental procedure. This prevented the use of slow onset and duration agonists. It would have been interesting to measure the effects of insulin in this model after long duration of perfusion for e.g. 4 hours in order to parallel the studies of that Baron and Steinberg that were carried out on human subjects.

The blood pressure cuff provided a good model for non-invasive measurement of endothelial function for use in chronic studies. Unfortunately, this method could not be used in any further experiments due to the pigmentation of the Zucker rat's tail. However, the tail cuff has been tested and is ready for use in future studies in other rodent models.

The existence of oxidant stress, endothelial function and insulin resistance was investigated in the pre-diabetic obese Zucker rat. As the insulin resistant syndrome is only in its early stages in the 13 week-old obese Zucker rat, the extent of oxidant stress and endothelial

dysfunction was uncertain. The presence of oxidant stress, endothelial function and insulin resistance was investigated prior to characterising the relationship between them and their involvement in disease progression by the use of pharmacological intervention.

↑ Fasting glucose	↑ Fasting insulin
↓ Glucose disposal	↑ 8-epi-PGF _{2α}
↑ Endothelial function	↑ Nitrate/nitrite
↑ Blood pressure	↓ Blood volume

Table 8.1 Characterization of insulin resistance, endothelial function and oxidant stress in the 12-13 weeks-old obese Zucker rat.

Oxidant stress, endothelial hyperfunction and insulin resistance were exhibited in the 12-13 week-old obese Zucker rat without any pharmacological intervention (Table 8.1). These results together could suggest enhanced oxidant stress contributes to insulin resistance and compensatory enhanced endothelial function. This may happen through damage to the insulin receptors or their signalling pathways, for example by the production of AGE or NAD(P)H-redox imbalances. Endothelial hyperfunction *ex vivo* may be a compensatory mechanism to the increased O₂[•] present *in vivo* in this obese rat. However, the fasting glucose in the obese Zucker rat was increased compared to the lean, but was within a normal range of <6mM and was therefore not strictly hyperglycaemic. Hyperglycaemia-induced damage to insulin receptors and the endothelium is therefore unlikely.

Alternatively, together these results could suggest that insulin resistance contributes to the enhanced endothelial function as a part of a compensatory mechanism, which counteracts the poor glucose disposal – hence minimal fasting hyperglycaemia in the obese Zucker rat. In addition, the insulin resistant syndrome could lead to oxidant stress through increased levels of post prandial plasma glucose. The insulin resistant syndrome is usually associated

with increased blood pressure and poor endothelial function *in vivo* due to an increased sympathetic output. This would in turn increase the need for enhanced endothelial function. An enhanced sympathetic drive would also reduce the observed insulin and NO -derived vasodilation as it would antagonise the balance in preference of constriction. Studies using the euglycaemic clamp technique support this notion, and one review (Fagius, 2003) showed that in healthy subjects, insulin infusion to levels corresponding to a postprandial state caused a sustained moderate increase in muscle sympathetic nervous activity. A peripheral vasodilation was also observed despite the enhanced sympathetic vasoconstrictor command; blood pressure remained stable (Anderson *et al.*, 1991). Another, similar study described supraphysiological insulin levels that induced an increase in muscle sympathetic nervous activity, which was also without a hypertensive reaction (Berne *et al.*, 1992). Insulin mediated vasodilation on muscle vessels, exerted via NO release opposes the effect of sympathetic excitation and thereby explains the absence of any rise in blood pressure.

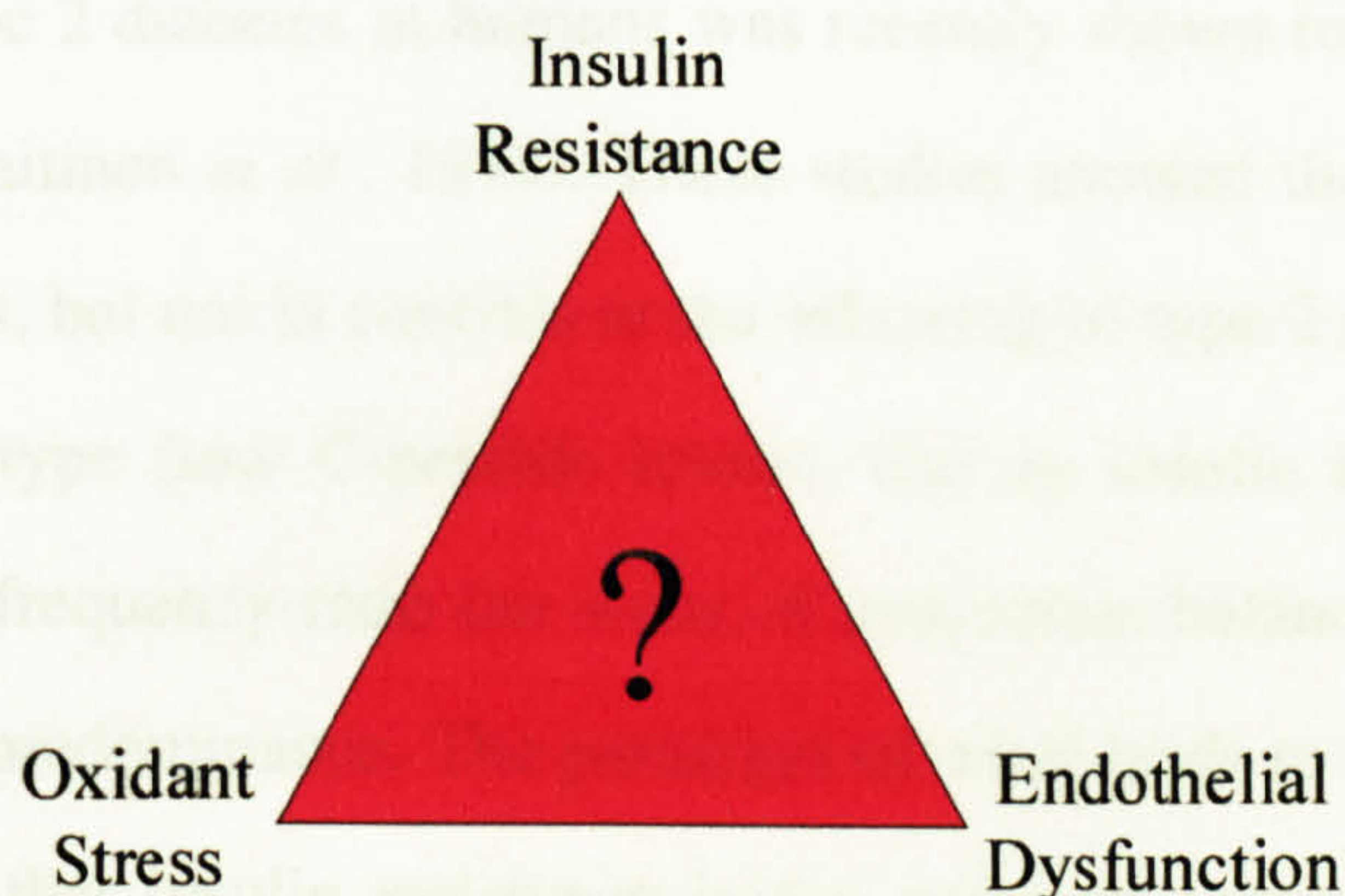


Figure 8.1 Paradigm of insulin resistance, endothelial function and oxidant stress.

Finally, although unlikely, these results could suggest that endothelial dysfunction may be responsible for insulin resistance. For example, the enhanced generation of NO may lead to sympathetic nervous system compensation and a balance tipped toward vasoconstriction

that will impair glucose uptake. In addition, if the NO pathway is constantly stimulated by other mediators, then insulin's small vasodilatory effect may be lost in the large overall vasodilation or balanced by sympathetic constriction. Impaired endothelial function can also lead to increased $O_2^{\cdot-}$ generation. It has been shown that the NOS enzyme can generate $O_2^{\cdot-}$ instead of NO when the cells lack cofactors such as arginine and BH_4 . This would most likely be when the cell is "over-worked" by the constant high generation of NO.

Studies of the obese Zucker rat suggest that the pathological progression is via central nervous system and autonomic nervous system dysfunction that results in insulin over secretion and dysregulation of insulin counter regulatory hormones. Certainly, this compares with studies in young 6-7 week-old obese rats, where levels of insulin were approximately 3 fold higher compared to the lean rats. These obese rats' glucose disposal was impaired too (Ionescu *et al.*, 1985). Evidence to suggest the involvement of autonomic dysfunction in early type 2 diabetes in humans was recently shown in heart rate variability studies using E.C.G (Laitinen *et al.*, 1999). These studies showed that in the offspring of insulin resistant subjects, but not in controls or the offspring of type 2 diabetic patients with insulin secretion phenotype (low C-peptide levels), that an insulin infusion significantly increased the low/high frequency ratio (an index of autonomic balance) to a higher value, indicating sympathetic predominance. The paradigm of what leads to this is still uncertain. It is, however, known that insulin resistance is the common factor in the syndrome X pathology, and that oxidant stress and endothelial dysfunction probably leads to the complications of the diabetic disease where they are not shown to be causative factors themselves.

Investigation of the effects of insulin on endothelium-dependent vasodilation in the hindquarters and aortic rings from the obese Zucker rat gave quite contrasting results. The hindquarters of the obese Zucker rat with already enhanced endothelial function showed

less vasodilation in the presence of insulin, whereas the aortic rings showed an enhanced relaxation in the presence of insulin. This tissue variability may help explain the selective action of insulin in the diabetic disease progression. The literature reports insulin mediates increased muscle blood flow in healthy subjects, a process that is impaired in type 2 diabetic patients (Steinberg *et al.*, 1996). This impaired action of insulin in the insulin sensitive tissues may be a factor in the early stage of disease in insulin resistant subjects; the macrovasculature at this stage may remain undamaged. It may be that macrovascular damage is the consequence of later disease and diabetic complications such as hypertension. Both my results from the investigation of the acute effect of insulin are from *ex vivo* preparations, therefore the increased blood pressure present in the obese Zucker rat is probably not due to hyperinsulinaemia itself as its aorta elicited insulin-induced dilation. This further suggests the involvement of enhanced sympathetic output. The use of a novel insulin bound NO donor drug would be useful to separate these sites of action and observe the whole effect *in vivo*.

It may be that ANS dysfunction is the initiator of the disease and that the pathophysiology of the obese Zucker rat arising from CNS disorders leading to insulin resistance could apply to humans. Diabetics have an insulin resistant, pre-diabetic state for some years prior to exhibiting type 2 diabetic disease. It follows therefore, similarly to the obese Zucker, that humans may require the “insult” from other environmental factors to push insulin resistance to frank diabetes; the increased processed food and sugar intake in our diet may progress the disease in those individuals susceptible.

Treatment with pharmacological tools such as pro-oxidants, antioxidants and fructose led to an understanding that environmental factors may be important, in that the changes in the oxidant balance caused modulation of both endothelial function and insulin resistance in the obese Zucker rat *in vivo*. As insulin resistance was affected too, it may be that oxidant

stress can be a causative factor in the progression from insulin resistance to type 2 diabetes at least in this model. This relatively new model of type 2 diabetes (pro-oxidant treated Zucker rat) showed the influence of oxidant status to change both endothelial function and insulin resistance together, suggesting there may be a relationship between them.

The antioxidant treatment gave positive evidence for the use of vitamin E in the early stages of diabetes, possibly by slowing the progression of the disease. Although this area of vitamin intake is extremely controversial, there is evidence of insulin depleting vitamin E supplies in circulating lipids regardless of the presence of insulin resistance (Galvan *et al.*, 1996). It would therefore follow that in hyperinsulinemic states, increased vitamin E would be used. This data sits alongside other evidence for the benefits of antioxidant therapy in type 2 diabetes, whilst the physiological and pathological balance of the oxidant status remains debatable.

As for the protective effect by fructose in these studies, it can only be said that there was no increase in oxidant stress. Therefore impairment of endothelial function and worsened insulin resistance would not have been expected. However, the mechanism by which fructose induced protection is unclear. Increased triglycerides as a protective mechanism would contradict the fatty acid-induced insulin resistance hypothesis (Goldstein, 2002), as increased insulin resistance would have been expected. A feasible mechanism is that fructose forms stable complexes with Fe^{3+} and prevents the Fe^{2+} catalysed formation of OH^\bullet via the Fenton reaction. This would prevent increased oxidant stress and therefore decrease the pro-oxidant-induced toxicity. These results reinforce that notion of an oxidant balance as a fructose diet has been shown to increase oxidant stress in normal animals and yet can protect in diabetic disease.

In summary, I developed models for the assessment of endothelial function that are especially useful in diabetes research – the *in situ* perfused hindquarters and an *in vivo* ear blood flow model. Endothelial function and oxidant stress were then investigated in the obese Zucker rat – a model of insulin resistance. Subsequently, endothelial dysfunction and oxidant stress were found to coexist with insulin resistance in this 13 week-old pre-diabetic rat model. My studies went on to show that oxidants not only contribute to endothelial function and therefore the complications associated with type 2 diabetes, but that they may also be a causative factor in progression to frank type 2 diabetic disease. Finally, dietary insult with fructose showed, paradoxically, protection against oxidant-induced damage in the obese animal with pre-diabetes. These studies have shown that the oxidant balance is finely tuned (especially in the case of dietary fructose) and that much research is required before it is understood if oxidant stress, and its resultant damage to endothelial function, together with its aggravation of insulin resistance, is a significant cause of this very prevalent disease – type 2 diabetes.

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